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TOWARD A MOLECULAR UNDERSTANDING OF HALLUCINOGEN ACTION

A Thesis

Submitted to the Faculty

of

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by

Jason Charles Parrish

In Partial Fulfillment of the

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of

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For Adriane

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PREFACE

The concept of ultimate reality has driven many great minds to madness. Of those that have survived this fate, from the great philosophers of antiquity to our current theoretical physicists, no one has explained the nature of reality to the satisfaction of the persistent questioner. The destruction of past civilizations and their accumulated knowledge by war and natural disasters has probably contributed to the relatively slow development of our understanding. Perhaps it is not possible for the human mind to understand fundamental reality, although, why so many people have the impulse to continue questioning even with this realization must give us pause.

The 20th century great minds of quantum physics proposed many models of ultimate reality from Copenhagen interpretation of Niels Bohr to the many worlds hypothesis of Hugh Everett. Unfortunately, these scientists came to the realization that quantum mechanics alone could not describe the nature of fundamental reality. All of these models ran into the problem of human consciousness or "the measurement problem" barrier. It is this problem that links the study of human consciousness with the study of theoretical physics.

Some believe that we can understand human consciousness at the neural systems or even the molecular level. An interesting logical result of this belief is

that if consciousness can be explained fully by a combination of molecular phenomena, then consciousness must follow the laws of chemistry and physics. This result eliminates the possibility of humans possessing what is referred to as conscious free will. If consciousness is a molecular phenomenon then humans, like molecules, must react specifically to a given set of initial conditions. Molecules can not have conscious free will because their actions follow a defined set of laws. Conscious free will becomes an illusion made manifest by the complexity of the variables before and after a reaction to a stimulus occurs. The study of consciousness now becomes the study of complex reactions to external and internal stimuli.

For many people, hallucinogenic drugs alter reality perception. Likewise, people with what are today considered mental disorders often have deficits in reality perception. Many mental disorders are thought to involve transient or persistent changes in neurotransmitter equilibrium substantially similar to the effects of hallucinogenic drugs. The study of the neural and molecular mechanisms behind mental disorders and the transient effects of hallucinogenic drugs has hinted at the regions of the brain that are involved with the standard perception of reality and the foundation of consciousness. It is the author's hope that this work contributes to our further understanding of fundamental reality.

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LIST ABBREVIATIONS

2-AG	2-arachidonoylglycerol
5-HT	5-hydroxytryptamine
AA	arachidonic acid
ADT	agonist-directed trafficking
AEA	anandamide
ASC	altered state of consciousness
βARK	β-adrenergic receptor kinase
CB1	cannabinoid receptor 1
COX	cyclooxygenase
CSTC	cortico-striato-thalamo-cortical
DAG	diacylglycerol
DEA	Drug Enforcement Administration
DGL	diacylglycerol lipase
DMT	*N,N*-dimethyltryptamine
DSE	depolarization-induced suppression of excitation

DSI	depolarization-induced suppression of inhibition
EPSC	electrically-evoked post-synaptic current
ESR	ear scratch response
FAAH	fatty acid amide hydrolase
GABA	gamma amino butyric acid
GPCR	G-protein coupled receptor
HTR	head twitch response
IP_3	inositol triphosphate
LC	locus coeruleus
LSD	lysergic acid diethylamide
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MGL	monoacylglycerol lipase
NAPE	N-arachidonoyl phosphatidylethanolamine
NAT	N-acyltransferase
OCD	obsessive-compulsive disorder
PA	phosphatidic acid
PAH	phosphatidic acid hydrolase

PC	phosphatidylcholine
PET	positron emission tomography
PIP_2	phosphatidylinositol diphosphate
PKC	protein kinase C
PLA_2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PtBu	phosphatidylbutanol
PTX	pertussis toxin
RGS	regulator of G-protein signaling
TRPC	transient receptor potential C
VR1	vanilloid receptor 1
WDS	wet dog shakes

ABSTRACT

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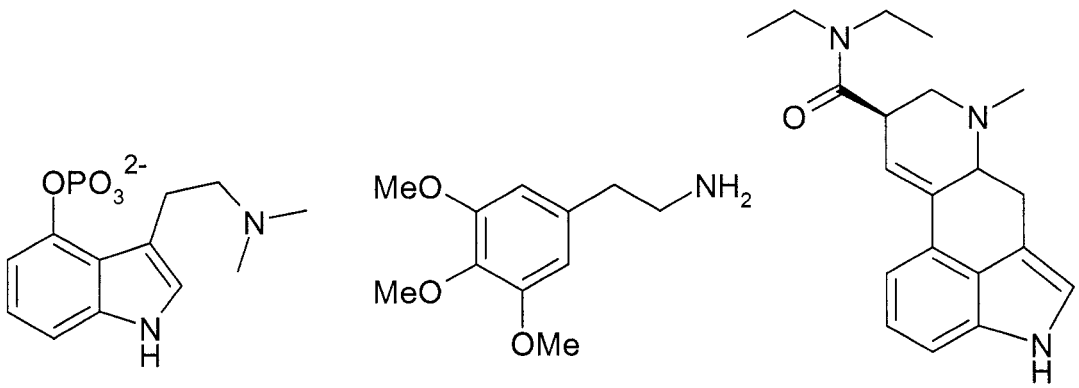
The serotonergic hallucinogens are a diverse group of chemicals that have in common their affinity for the mammalian 5-HT_{2A} receptor. Biochemical studies have demonstrated that the 5-HT_{2A} receptor couples to many signaling pathways, forming a complex signaling network that affects various cellular processes. The purpose of this study was to investigate 5-HT_{2A} receptor-mediated signal transduction in an attempt to identify signaling events that are unique to hallucinogens. The first study was initiated in order to explore the effect on 5-HT_{2A} receptor-dependent phospholipase C activation of adding a methyl group to the alpha position of the side chain of a small series of phenethylamine agonists. Because the alpha methyl group renders the phenethylamines chiral, the effect on phospholipase C activation of both (*R*)- and (*S*)-enantiomers also was assessed. Next, the discovery of 5-HT_{2A} receptor-dependent 2-arachidonoylglycerol release is presented, along with a characterization of the signaling pathways that are involved with the production and hydrolysis of this important endocannabinoid. The production and release of 2-arachidonoylglycerol was demonstrated to be dependent on phospholipase C with no contribution coming from the 5-HT_{2A} receptor-dependent phospholipase

D pathway. Finally, in light of the fact that 5-HT_{2A} receptor-dependent 2-arachidonoylglycerol release was demonstrated to be phospholipase C-dependent, the pathway for the biosynthesis of the major hydrolysis product of 2-arachidonoylglycerol, arachidonic acid, was examined. Results indicate that the production and release of arachidonic acid in the cell line tested is dependent on both fatty acid amidohydrolase-catalyzed hydrolysis of 2-arachidonoylglycerol and sn2-selective diacylglycerol lipase-catalyzed hydrolysis of diacyglycerol derived from sequential phospholipase D and phosphatidic acid hydrolase activation. The data presented herein contribute to our understanding of 5-HT_{2A} receptor-mediated signal transduction and also help to explain the results of animal studies that have demonstrated a role for endocannabinoids in certain behavioral effects that are mediated by 5-HT_{2A} receptor activation. Given the role that the 5-HT_{2A} receptor plays in normal and aberrant cognition, a detailed understanding of its molecular pharmacology is essential for the development of drugs that modulate its function, in order to alleviate the symptoms of central nervous system disorders such as schizophrenia, obsessive compulsive disorder, depression, and others.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

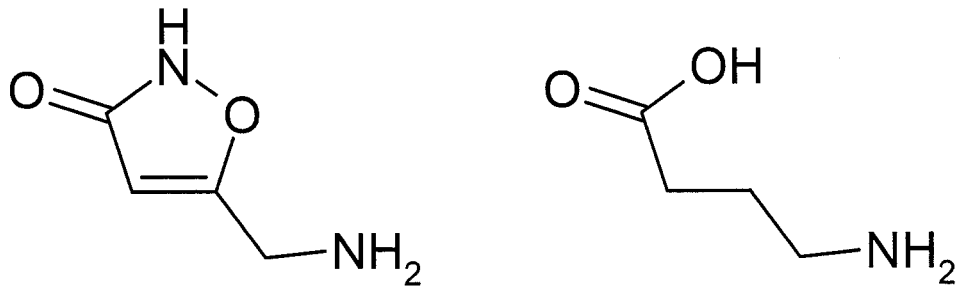
1.1. Hallucinogens: History of use

Although diverse in chemical structure, the hallucinogens (Fig 1.1) elicit a relatively consistent altered state of consciousness (ASC) in humans that has qualitatively measurable psychological parameters (Dittrich 1998). Drug-induced ASCs are speculated to have been familiar to humans for more than 6000 years and may have played a role in the development of early civilizations (Nichols and Chemel, 2006). For example, the basidiomycete *Amanita muscaria*, brought to the Indian subcontinent by Aryan invaders many thousands of years ago, produces both the amino acid ibotenic acid and its spontaneous decarboxylation product muscimol, a structural homologue of the inhibitory neurotransmitter GABA (Fig. 1.2). The illustrious ethnomycologist R. Gordon Wasson speculated that this psychoactive mushroom was the mythical Soma of the *Rig Veda* (Wasson 1968;Wasson and Ingalls 1971). Another example from the ancient world is the ergoline-containing $\kappa\upsilon\kappa\varepsilon o\nu$ (kykeon) brew of the ancient Greek Eleusian Mysteries, thought to have been prepared from grain that had been parasitized by the ergot fungus (Wasson *et al.* 1978). Examples from the New



Psilocybin **Mescaline** **LSD**

Figure 1.1 Representative hallucinogens from each structural class. Psilocybin and mescaline are well known naturally occurring tryptamine and phenethylamine hallucinogens, respectively. LSD, however, is a semi-synthetic ergoline hallucinogen.



Muscimol **GABA**

Figure 1.2 The structures of Muscimol and GABA. Muscimol is essentially a rigid analogue of GABA as can be readily observed when their two-dimensional representations are compared.

world with a long history of traditional use include the ergoline-containing *Ololiuqui* (*Rivea corymbosa)* (Schultes 1941), and the mescaline-containing Peyote cactus *Lophophora williamsii* (Heffter 1898;Perrine 2001). Indeed, Peyote use can be traced back more than three thousand years (Schultes and Hofmann 1992). Although the widespread psilocybin-producing basidiomycetes of the *Psilocybe* genus were used by peoples of the New world to induce ASC (Ott and Bigwood 1978;Schultes and Hofmann 1979;Schultes and Hofmann 1992), it is likely that, given their cosmopolitan nature, they were used by prehistoric people of all continents. Use of hallucinogenic plants and fungi by peoples of pre-antiquity may have contributed to the supernatural underpinnings that form the basis of all proto-religions and the deities that represent them (Nichols and Chemel, 2006).

Today religious use of hallucinogens continues. *N,N*-Dimethyltryptamine (DMT) is found in the ayahuasca sacrament of the Brazilian UDV church (McKenna *et al.* 1984;McKenna and Towers 1984;Callaway *et al.* 1996;Grob *et al.* 1996;Riba *et al.* 2002;Yritia *et al.* 2002;Riba *et al.* 2003). The mescaline-containing Peyote also is currently used as a sacrament by the Native American Church (Stewart 1987). The relationship between hallucinogens and profound religious experience may not be entirely speculative. A very recent double-blind placebo-controlled study clearly demonstrated that psilocybin can produce effects indistinguishable from to spontaneously-occurring mystical states (Griffiths *et al.* 2006). With a long history of use for spiritual and medical purposes, hallucinogens have a relatively low incidence of adverse reactions

(Strassman 1984;Halpern and Pope, Jr. 1999), demonstrate little or no addiction potential (O'Brien 2001), and presently have retrograde governmental regulations (Strassman 1991) that effectively hinder the pursuit of a molecular understanding of consciousness.

1.2. Legal status in the United States

Hallucinogens are officially classified by the Drug Enforcement Administration (DEA) as Schedule I controlled substances. In order to be classified as a Schedule I controlled substance the drug or other substance must meet a three-pronged test: (A) exhibit a high potential for abuse, (B) must have no currently accepted medical use in treatment in the United States, and (C) there must be a lack of accepted safety for use of the drug or other substance under medical supervision. Currently, of the three classification parameters used by the DEA only (B) is true of the hallucinogens, although the Schedule I classification essentially prevents research that might negate (B) above. Past clinical and case studies, as well as anecdotal reports, have provided evidence for the medical utility of hallucinogens in treating addiction (Mangini 1998) and obsessive compulsive disorder (OCD) (Savage *et al.* 1962;Brandrup and Vanggaard 1977;Leonard and Rapoport 1987;Hanes 1996;Moreno and Delgado 1997;Delgado and Moreno 1998a;Delgado and Moreno 1998b). In the United States possession of any quantity of a hallucinogenic substance by unlicensed individuals constitutes a federal felony. Ironically, the discovery of DMT in cerebrospinal fluid (Gillin *et al.* 1976;Corbett *et al.* 1978;Barker *et al.* 1981)

technically criminalized most of the US population. Despite the enormous legal barriers to research with hallucinogens, many notable investigators have devoted their lives to the study of this most interesting class of drugs.

1.3. Psycho-physiological model of hallucinogen action

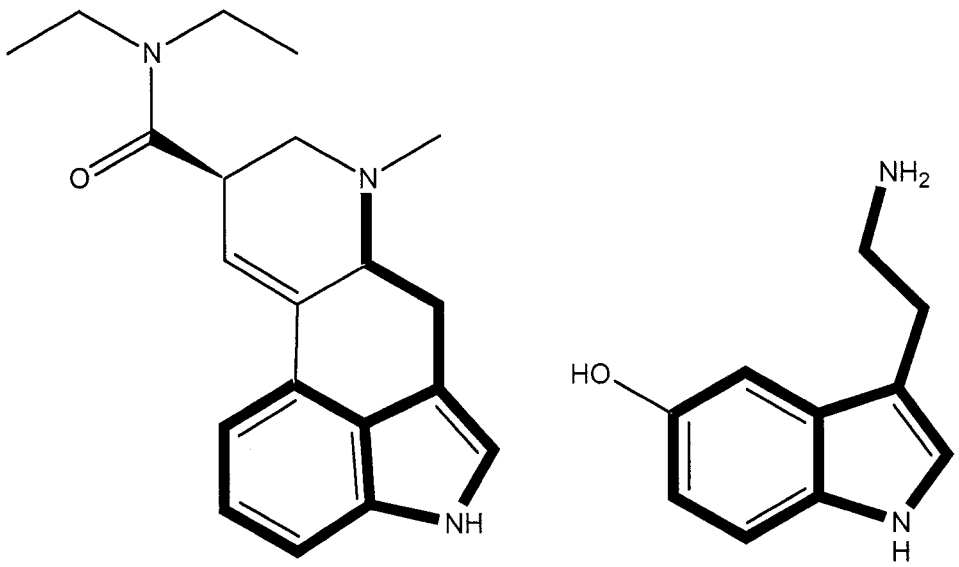
Hallucinogenic drugs can produce an altered perception of the self construct that has parallels to many of the symptoms of schizophrenia. It is for this reason that early research with hallucinogens attempted to use them as models for psychosis. With evidence that hallucinogens can model acute schizophrenia in humans, the past decade has seen a revitalization of the hallucinogen-induced psychosis model (Vollenweider 1998). ASC, measurable with current psychological protocols (Dittrich 1998) and with brain imaging studies (Vollenweider *et al.* 1997) has allowed the possibility for the study and comparison of "normal" waking conscious states with ASC. For this reason, hallucinogens have been used to test hypotheses concerning the inter-neuronal signaling architecture involved in both normal waking and ASC (Vollenweider *et al.* 1997;Vollenweider 1998;Vollenweider *et al.* 1998;Vollenweider and Geyer 2001).

Vollenweider and Geyer (2001) propose a model for the mechanism of hallucinogen action at the neuronal systems level. This mechanism involves opening what is referred to as the thalamic filter or gate of cortico-striato-thalamo-cortical (CSTC) feedback loops. According to this model, the thalamic filter can be "opened" by hallucinogens interacting with serotonin2A (5-HT_{2A})

receptors on GABAergic neurons in the striatum, leading to over stimulation of the cortex and a reduction in its ability to maintain cognitive homeostasis. Under normal conditions, inhibitory input to the thalamus from the striatum is increased with cortical feedback, preventing over-excitation of the cortex. As predicted by their model, hyperactivity of the prefrontal cortex in humans was observed by positron emission tomography (PET) when the hallucinogen psilocybin was administered to volunteers (Vollenweider *et al.* 1998). The neural mechanisms underlying other abnormal cognitive behaviors such as OCD (Delgado and Moreno 1998a;Delgado and Moreno 1998b), suicidality, anorexia nervosa, and major depression (De Angelis 2002) may also be caused by CSTC gating deficits induced by 5-HT_{2A} receptor-mediated signaling.

1.4. Serotonin receptors and hallucinogen action

Following the 1943 discovery of the hallucinogen LSD, structural similarities to 5-HT (Fig. 1.3), newly-identified and detected in the brain shortly thereafter, led early researchers to propose a serotonergic mechanism of action for hallucinogens. Perhaps even more important was the subsequent recognition that aberrant mental states might result from disturbances in brain chemistry. Although today we take that as a given, in that era it was a controversial thesis, with mental illness, and especially schizophrenia, more often attributed to poor parenting skills, especially by the mother. The early focus on the role and



LSD **5-HT**

Figure 1.3 The structural similarities of LSD and 5-HT. Both LSD and 5-HT contain a tryptamine pharmacophore illustrated here by the bold lines.

importance of serotonin in the brain almost certainly accelerated the development of the then-fledgling area of neuroscience. It is entirely possible that modern medications to treat depression and migraine, for example, might not have been developed as quickly, or even found at all, had LSD not been discovered.

It was soon demonstrated that LSD antagonized the effects of 5-HT in peripheral tissues (Gaddum 1953), however, it was later demonstrated that LSD had direct agonist activity at 5-HT receptors in the CNS (Anden *et al.* 1968). Within a decade of this latter discovery, other hallucinogens including psilocybin (psilocin), DMT, 5-MeO-DMT, and 2,5-dimethoxy-4-methylamphetamine (DOM) were demonstrated to have effects at 5-HT receptors consistent with an agonist mechanism of action (Freedman *et al.* 1970;Anden *et al.* 1971;Randic and Padjen 1971;Fuxe *et al.* 1972;Leonard 1973;Anden *et al.* 1974).

Since the initial discovery of the agonist mechanism of action for hallucinogens, serotonin receptors of different subtypes have been found expressed throughout the mammalian organism (Hoyer *et al.* 2002). All 14 subtypes of 5-HT receptors identified to date, with the exception of the 5-HT_3 pentameric ligand gated ion channel, have been determined to be heptahelical G-protein coupled receptors (GPCRs). Of these 5-HT receptor subtypes, the 5-HT_{2A} receptor has been identified as the most critical for hallucinogen action.

1.5. Serotonin 5-HT_{2A} receptors

As mentioned earlier, agonist activation of the 5-HT_{2A} receptor is a required element in the hallucinogen mechanism of action. Perhaps the most convincing evidence for this claim comes from studies where the hallucinogenic effects of psilocybin were blocked in human subjects preadministered ketanserin, a relatively selective 5-HT_{2A} receptor antagonist (Vollenweider *et al.* 1998;Carter *et al.* 2005).

Serotonin 5-HT_{2A} receptors are found in many cell types within the CNS, with particularly high densities in the neurons of the neocortex (Hoyer *et al.* 1986;Wright *et al.* 1995;Willins *et al.* 1997;Miner *et al.* 2003). Within the neocortex, 5-HT_{2A} receptors are found on apical dendrites of pyramidal cells and on parvalbumin-costaining GABAergic interneurons, especially in layer V (Willins *et al.* 1997). Serotonin 5-HT_{2A} receptor mRNA also has been identified in the reticular, lateral geniculate, anterodorsal, and ventromedial nuclei of the thalamus (Pompeiano *et al.* 1994).

In the locus coeruleus (LC), serotonin 5-HT_{2A} receptor agonists indirectly cause a decrease in spontaneous firing of LC neurons, although at the same time increase the sensitivity of the LC neurons to sensory inputs (Aghajanian 1980;Rasmussen *et al.* 1986). This action has the effect of increasing the signal to noise ratio for sensory inputs into the LC (Woodward *et al.* 1979). Although 5-HT_{2A} receptors have not been found on LC neurons, the observed 5-HT_{2A} receptor-mediated decrease in spontaneous LC firing was blocked by the local infusion of GABA antagonists (Chiang and Aston-Jones 1993), suggesting that 5-

HT_{2A} receptors located on GABAergic interneurons within the vicinity of the LC were facilitating the effect. The 5-HT_{2A} receptor-mediated increase in sensitivity to sensory inputs was blocked by local infusion of an NMDA receptor antagonist into the LC, supporting a role for a selective increase in excitatory input from unidentified glutamatergic innervations modulated by 5-HT_{2A} receptor activation (Chiang and Aston-Jones 1993).

Within the cortex, hallucinogenic 5-HT_{2A} receptor agonists appear to increase excitatory postsynaptic currents (EPSCs) in layer V pyramidal cells (Aghajanian and Marek 1998) (Fig. 1.4). Many of the glutamatergic afferents that form asymmetric synapses with layer V cortical pyramidal cell dendrites originate from mediodorsal thalamic neurons. Chemically induced lesions of these thalamic projections decrease the frequency of serotonin-induced EPSCs by 60%, suggesting that the majority of 5-HT_{2A} receptor-dependent pyramidal cell excitability has a presynaptic mechanism of action (Marek *et al.* 2001). Microiontophoretically applied LSD was demonstrated to cause an increase in the asynchronous release of presynaptic glutamate onto pyramidal cells, providing further evidence for a presynaptic mechanism of hallucinogen action (Marek and Aghajanian 1998;Marek *et al.* 2001). The fact that 5-HT_{2A} receptor immunoreactive sites (Jakab and Goldman-Rakic 1998;Miner *et al.* 2003) and 5-HT_{2A} receptor mRNA (Pompeiano *et al.* 1994) are not typically observed in mediodorsal thalamic neurons supports the hypothesis that hallucinogens bind

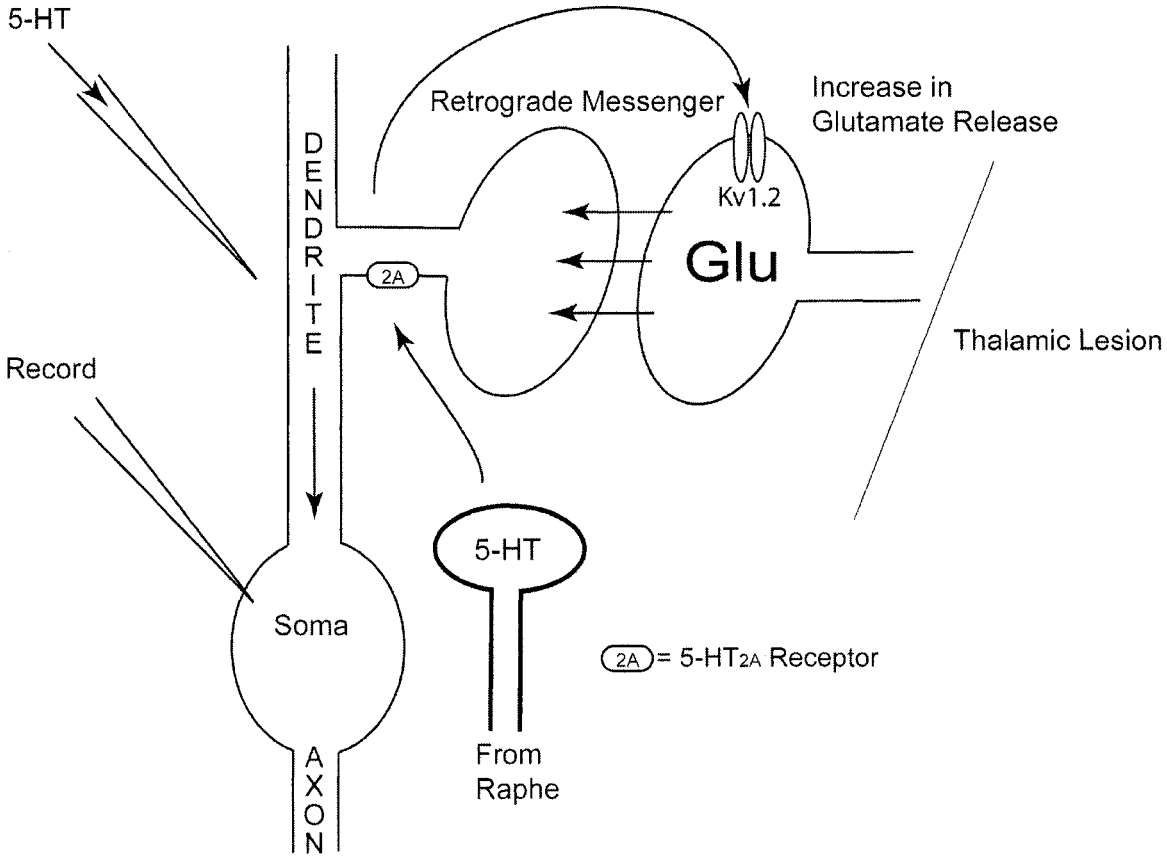


Figure 1.4 A summary of 5-HT_{2A} receptor-mediated presynaptic glutamate release. Following stimulation of the 5-HT_{2A} receptor, a hypothesized retrograde messenger diffuses to the presynaptic glutamatergic axon terminal facilitating asynchronous glutamate release by blocking Kv1.2 potassium channels. Approximately 60% of the glutamatergic axon terminals demonstrating this response are inhibited by thalamic lesions.

postsynaptically, causing the release of a retrograde messenger that diffuses to the presynaptic axon terminal where it mediates its effects on presynaptic glutamate release. In search of the hypothesized retrograde messenger, Lambe and Aghajanian (2001) reported that blocking presynaptic Kv1.2 channels with α-dendrotoxin occluded 5-HT_{2A} receptor-dependent presynaptic glutamate release at thalamocortical terminals in the prefrontal cortex. Both the endocannabinoid anandamide (Poling *et al.* 1996a) and the tetraeneoic arachidonic acid (AA) (Poling *et al.* 1996b) have been shown to block Kv1.2 channels, suggesting that endogenous eicosanoids mediate this process. Although stimulation of the 5-HT_{2A} receptor has been reported to cause the release of AA (Felder *et al.* 1990), 5-HT_{2A} receptor-dependent endocannabinoid production has thus far not been demonstrated.

1.6. Serotonin 5-HT_{2A} receptor signal transduction

At the molecular level, the 5-HT_{2A} receptor couples to $G_{q/11}$ heterotrimeric G-proteins, promoting the PI-PLC-dependent production of the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) (Conn and Sanders-Bush 1984;Conn and Sanders-Bush 1985;Conn *et al.* 1986) (Fig. 1.5). IP_3 interaction with receptors on the endoplasmic reticulum causes calcium release from intracellular stores, whereas membrane-associated DAG has other

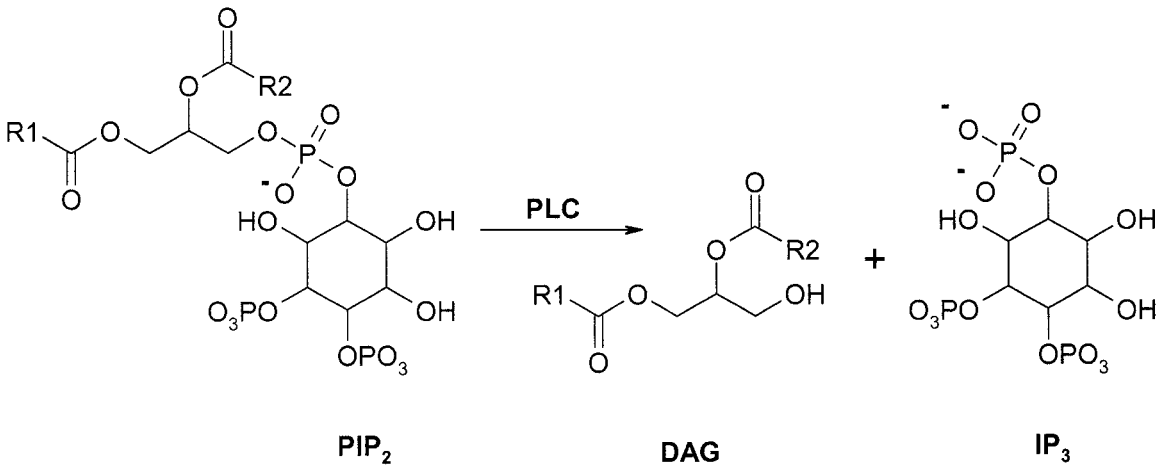


Figure 1.5 PLC-catalyzed hydrolysis of phosphatidylinositol (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3).

fates. For example, DAG can stimulate the influx of additional calcium by interacting with plasma membrane TRPC3/6/7 cation channels (Trebak *et al.* 2003a;Trebak *et al.* 2003b), may activate protein kinase C (PKC) (Nishizuka 1992), or act as a substrate for DAG lipases (DGL)s (Bisogno *et al.* 2003). Calcium, effectively functioning as a third messenger, controls a wide range of cellular processes (Berridge 1993) including the activation of calmodulin and downstream effectors.

The 5-HT_{2A} receptor also promotes the activation of phospholipase D (PLD) and the production of phosphatidic acid (PA) by coupling directly with the monomeric G-proteins ARF1 and ARF6 (Robertson *et al.* 2003) through an interaction with the C-terminal domain of the receptor. ARF1 mediates transport in the early secretory pathway from the Golgi to the endoplasmic reticulum (ER) (D'Souza-Schorey and Chavrier 2006), whereas plasma membrane-bound ARF6 is implicated in the regulation of G-protein-coupled receptor internalization (Houndolo *et al.* 2005). PLD catalyzes the production of PA that is further metabolized to the second messenger DAG by the enzyme phosphatidic acid hydrolase (PAH) (Fig. 1.6) (Perry *et al.* 1992;Bisogno *et al.* 1999). The fact that the 5-HT_{2A} receptor can directly couple to two different G-proteins, namely $G_{q/11}$ and ARF1/6, allows for the possibility of agonists with different structural motifs differentially to stimulate different signaling pathways, leading to unique

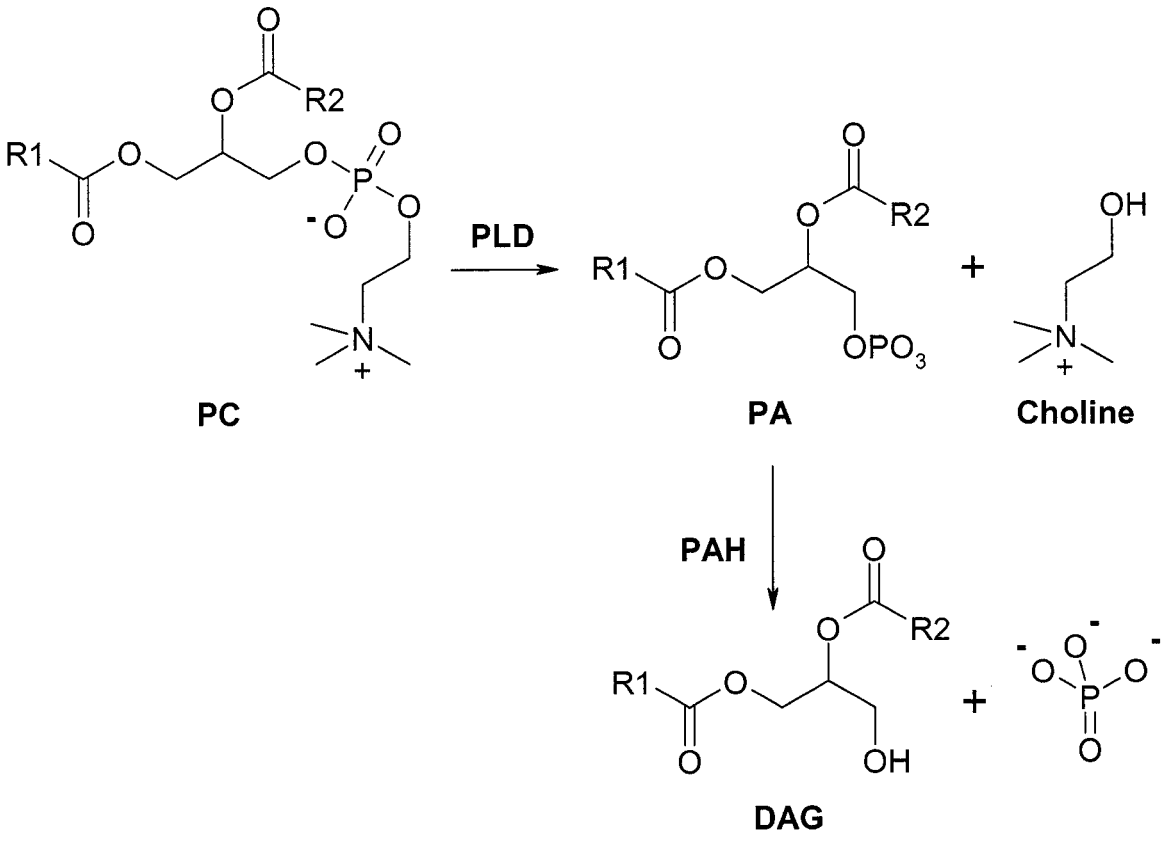


Figure 1.6 The PLD pathway. PLD catalyzes the hydrolysis of phospholipids. PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. In a subsequent reaction, phosphatidic acid hydrolase catalyzes the hydrolysis of PA to DAG and free phosphate.

signaling events. As will be discussed at the end of this thesis, this type of agonist-directed trafficking (ADT) (Kenakin 1995) may be what ultimately generates the subjective effects of hallucinogens in humans.

In addition to $G_{q/11}$ and Arf1, 5-HT_{2A} receptor stimulation has been shown to activate the monomeric G-protein RhoA, p38 mitogen-activated protein kinase (MAPK), and p42/44 MAPKs, promoting the extracellular release of eicosanoids (Kurrasch-Orbaugh *et al.* 2003a). Studies in NIH3T3 cells stably expressing the rat 5-HT_{2A} receptor (NIH3T3-5HT_{2A} cells) also support the notion that $G_{12/13}$ and $G_{i/o}$ heterotrimeric G-proteins are involved in 5-HT_{2A} receptor-dependent eicosanoid release. Evidence of $G_{12/13}$ activation comes from two key experiments. Transient expression of the N-terminal RGS homology domain of RhoGEF led to a decrease in measured [^{3}H]-eicosanoid release. The N-terminal RGS homology domain of RhoGEF binds to $G_{12/13}$ type G-proteins, preventing them from associating with and activating downstream effectors. Additionally, covalently inactivating RhoA with C3 toxin also resulted in a decrease in measured [^{3}H]-eicosanoid release (Kurrasch-Orbaugh *et al.* 2003a). Together, these results support the hypothesis that the 5-HT_{2A} receptor activates $G_{12/13}$ type G-proteins leading to the production of eicosanoids. Evidence for the 5-HT_{2A} receptor dependent activation of $G_{i/o}$ G-proteins comes from the fact that pertussis toxin (PTX) inhibited 5-HT_{2A} receptor-dependent [^{3}H]-eicosanoid release by 60% while having no effect on 5-HT_{2A} receptor-dependent IP accumulation. Additionally, sequestration of $G_{\beta\gamma}$ with the C-terminal end of β-adrenergic receptor kinase (βARK_{CT}) inhibited 5-HT_{2A} receptor-dependent [^{3}H]-

eicosanoid release by 50%, while having no effect on 5-HT_{2A} receptor-dependent IP accumulation. Inhibition of the downstream effectors p38 MAPK with SB202190 or MEK1,2 with PD098059 decreased 5-HT_{2A} receptor-dependent [^{3}H]-eicosanoid release by 50%, while having no effect on 5-HT_{2A} receptor-stimulated IP accumulation (Kurrasch-Orbaugh *et al.* 2003a). Taken ltogether, the results of Kurrasch-Orbaugh *et al.*, (2003a) show that [^{3}H]-eicosanoid release in NIH3T3-5HT2A cells is complicated, involving multiple 5-HT_{2A} receptor-coupled signaling pathways.

In summary, activation of the 5-HT_{2A} receptor dramatically changes the relative concentrations of membrane lipids in many ways (Fig. 1.7). For example, $G_{q/11}$-dependent activation of PI-PLC temporarily increases net DAG, while temporarily decreasing the concentration of phosphatidylinositol (PI). Arf1/6 and RhoA-dependent PLD activation temporarily decreases the concentration of phosphatidylcholine, while increasing the concentration of phosphatidic acid (PA). MAPK-dependent PLA_2 activation also temporarily decreases the concentration of PC, while increasing the concentration of both lysophosphatidylcholine and AA. Although many of these changes in lipid composition are associated with membrane budding and fusion events, some of them are associated with cell to cell communication. For example, the hydrolysis product of DAG, 2-arachidonoylglycerol (2-AG) has been demonstrated to be a

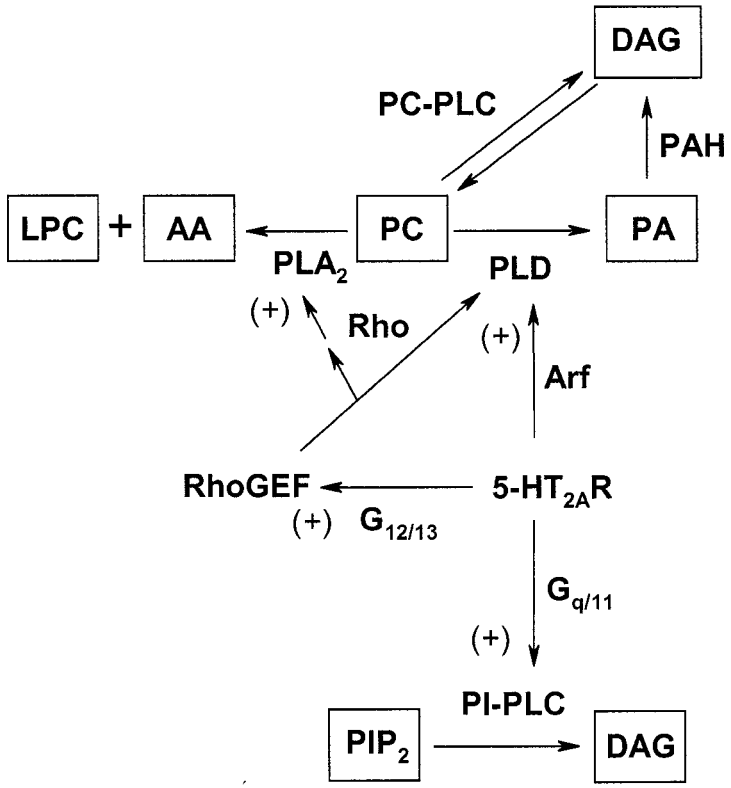


Figure 1.7 Summary of 5-HT_{2A} receptor-mediated lipid metabolism. Lipids are shown boxed whereas enzymes that modify them are illustrated with arrows that indicate the direction of the reaction under stimulated conditions. A (+) sign indicates a stimulation event.

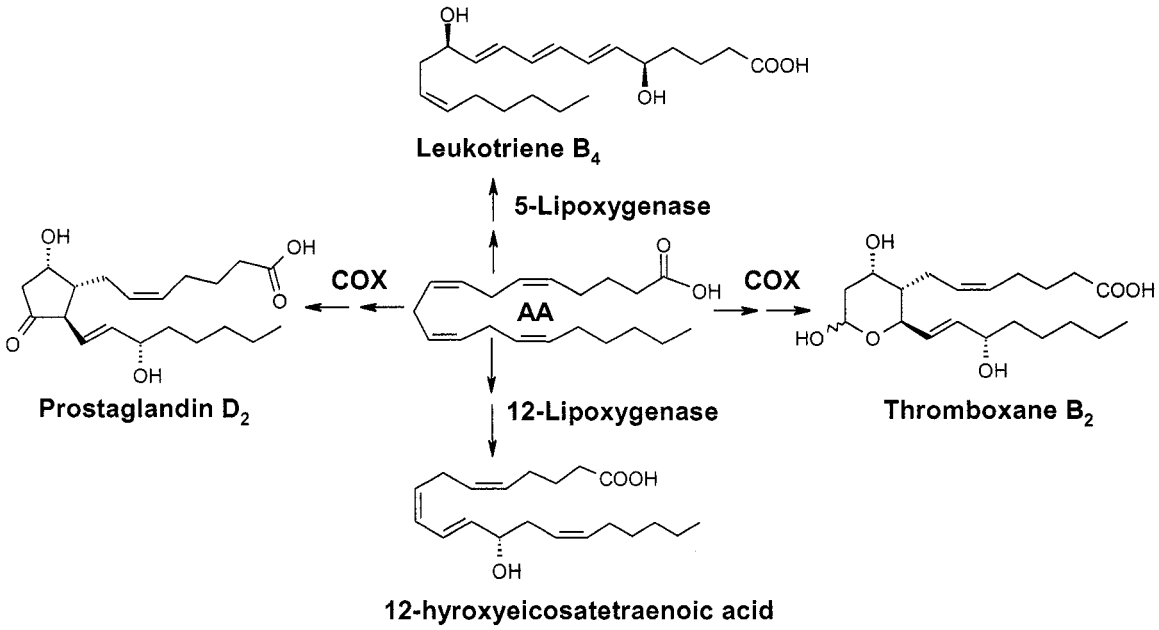


Figure 1.8 Four of the many metabolites of AA. AA is the substrate for many enzymes including cyclooxygenase (COX), lipoxygenase, and P450. The four metabolites illustrated here have all been demonstrated to be produced following 5-HT_{2A} receptor stimulation (Felder *et al.* 1990).

retrograde messenger at many types of neuronal synapses (Melis *et al.* 2004;Galante and Diana 2004;Maejima *et al.* 2005). Additionally, proinflammatory oxidation products of AA, including hydroxyeicosatetraenoic acids, thromboxane B_2, prostaglandins, and leukotrienes (Fig. 1.8) have all been shown to be released in mixed neuronal and glial cultures in a 5-HT_2 receptor-dependent way (Felder *et al.* 1990). Considering the evidence for the existence of a retrograde signaling molecule involved with 5-HT_{2A} receptor-mediated effects on glutamatergic afferents in the cortex, and the ability of both anandamide and AA to block Kv1.2 potassium channels, the signaling pathways involved with 5-HT_{2A} receptor-dependent eicosanoid release appear to be likely components in the hallucinogen mechanism of action. The last two specific aims within this thesis investigate the eicosanoid release pathways in more detail than previously reported.

1.7. Structural classes of hallucinogens

The hallucinogens can be classified into at least three definitive structural classes including the tryptamines, ergolines, and phenethylamines (Fig. 1.1). All three structural classes are both naturally-occurring and synthetic in nature, with some 200+ potential compounds known.

1.7.1. Tryptamine hallucinogens

Tryptamine hallucinogens have relatively high affinity for 5-$HT_{2A/2C}$ and 5-HT_{1A} receptors and are the closest structural analogues to 5-HT, a tryptamine itself. Possibly the most common hallucinogen in the biosphere is the tryptamine DMT. Relatively simple in structure, DMT has been identified in a considerable number of organisms, including species in the plant, animal, and fungi kingdoms. The discovery of DMT in human cerebrospinal fluid has even led some researchers to speculate that it may play a role in schizophrenia (Gillin *et al.* 1976;Corbett *et al.* 1978;Barker *et al.* 1981). DMT is not orally active, being rapidly deaminated by monoamine oxidase (MAO) in the liver. Pretreatment with an MAO inhibitor, however, prevents this metabolism and renders DMT active by the oral route. Replacing the amine nitrogens with larger alkyl substituents also hinders oxidation and confers oral activity (Shulgin and Shulgin 1997). The 4-hydroxy analogue of DMT, psilocin, is an orally active tryptamine that is found in mushrooms of the *psilocybe, gymnopilus,* and *panaeolus* genra.

1.7.2. Ergoline hallucinogens

The ergoline hallucinogens have a relatively complex pharmacology. For example, the prototypical hallucinogenic ergoline, LSD, has significant affinity for 5-$HT_{1A/1B/1D/1F}$, 5-$HT_{2A/2C}$, 5-$HT_{5A/5B}$, 5-HT_6, 5-HT_7 (Peroutka 1994), dopamine D_1,and D_2 (Watts *et al.* 1995), and α_1 and α_2 adrenergic receptors (U'prichard *et al.* 1977). Detectable in doses as low as 0.025 mg, LSD is one of the most

biologically potent hallucinogens. Of the many structural analogues of LSD that have been examined, only the N(6)-alkyl derivatives afford more potent compounds than the parent molecule (Hoffman and Nichols 1985). Substitutions on the nitrogen of the amide functional group generally lead to less potent compounds (Huang *et al.* 1994;Monte *et al.* 1995) except in the case of the equipotent *S,S*-dimethylazetidine analogue, as measured by binding affinity and drug discrimination assays in rats (Nichols *et al.* 2002). Halogen substitutions in the 2-position of the indole ring afford molecules with antagonist properties that are devoid of LSD-like psychopharmacology (Cerletti and Rothlin 1955;Rothlin 1957). Although the ergolines have a complex molecular pharmacology, their ability to elicit subjective hallucinogenic effects in humans is likely a result of their interaction with 5-HT_{2A} receptors. One can argue that the ancillary pharmacological actions of LSD may be important for certain qualitative aspects of its effects, but in that debate it would still have to be concluded that activation of 5-HT_{2A} receptors was a necessary, but not necessarily sufficient condition.

1.7.3. Phenethylamine hallucinogens

Phenethylamine hallucinogens, the most 5-HT_2 receptor selective class, have significant affinity for 5-$HT_{2A/2C}$ receptors whereas, unlike the tryptamine and ergoline hallucinogens, they lack significant affinity for 5-HT_{1A} receptors. The only naturally-occurring phenethylamine hallucinogen discovered to date is mescaline. Mescaline, perhaps the least potent of the hallucinogenic phenethylamines, has served as the template for the design of numerous

phenethylamine analogues with increased hallucinogenic potency. The synthesis of the phenylisopropylamine analogue of mescaline, 3,4,5-trimethoxyamphetamine (TMA) was first reported in 1947 (Hey 1947). TMA had roughly twice the potency of mescaline in human subjects (Shulgin *et al.* 1961). Many ring-substituted phenethylamines and phenylisopropylamines have been prepared and tested for biological activity in the ensuing 60 years. Phenylisopropylamine analogues were consistently found to be more potent and have longer durations of action when compared to their corresponding phenethylamine analogues (Glennon *et al.* 1983). The reason(s) for the difference in hallucinogenic potency between phenethylamines and their phenylisopropylamine analogues has not been determined but there are several likely explanations. First, hydrophobicity increases with the addition of an alpha-methyl group to a phenethylamine, and brain penetration will likely be higher. The alpha-methyl also will hinder metabolic deamination. But these explanations are not adequate to explain the potency differences, and results from *in vitro* experiments suggest a receptor level cause.

One study has systematically examined the functional difference between phenethylamines and their corresponding phenylisopropylamine analogues. Using *Xenopus laevis* oocyte membranes transiently expressing the rat 5-HT_{2A} receptor, voltage clamp techniques were used to measure current generated in response to phenylalkylamine analogue pairs (Acuna-Castillo *et al.* 2002). The authors of this study found that phenethylamines were virtually non-efficacious, whereas their corresponding phenylisopropylamine analogues were weak partial

agonists. The authors proposed that an antagonist or weak partial agonist mechanism of action for hallucinogens might still be tenable. Again, using the same oocyte assay system, these workers reported that phenethylamines are antagonists at the transiently expressed rat 5-HT_{2A} receptor (Villalobos *et al.* 2003). Based on the results of their study, the authors concluded that the notion that phenylalkylamine hallucinogens are full or partial 5-HT_{2A} agonists is questionable. Conflicting with their report, however, is the fact that a 5-HT_{2A} receptor antagonist blocks the effects of a hallucinogen in man, (Vollenweider *et al.* 1998;Carter *et al.* 2005). The clinical studies clearly indicate that the *Xenopus* oocyte model may not reflect 5-HT_{2A} receptor-mediated signaling relevant to human psychopharmacology.

Although the phenethylamines are achiral, phenylisopropylamines have two optical isomers. Most biological targets, usually macromolecular proteins, are chiral, stereospecific, and/or stereoselective in their actions. The eudismic ratio between the more active eutomer and the less active distomer can vary from several-fold to several hundred-fold, depending on structural considerations at the ligand-receptor interface.

Characterization of the optical isomers of phenylisopropylamines became possible with the development of an efficient asymmetric synthesis for their production (Nichols *et al.* 1973). The (*R*)-isomers of phenylisopropylamines are more potent *in vitro* (Dyer *et al.* 1973;Cheng *et al.* 1974), in animal models (Harris *et al.* 1977;Young and Glennon 1996), and in their ability to induce hallucinogenesis in humans (Shulgin 1973;Shulgin and Shulgin 1991).

Additionally, radioligand competition assays have determined that *(R)*-isomers of hallucinogenic phenylisopropylamines have higher affinity than their corresponding *(S)*-isomers (Johnson *et al.* 1990).

Despite extensive study, questions remain concerning the pharmacology of phenylalkylamines and their effects on 5-HT_{2A} receptor-dependent PLC activation. For example, do the phenylisopropylamines in general have higher potencies or intrinsic activities relative to their phenethylamine analogues for 5-HT_{2A} receptor-dependent PLC activation? Do the *R*-enantiomers of phenylisopropylamines have higher intrinsic activities relative to their phenethylamine analogues for 5-HT_{2A} receptor-dependent PLC activation? The first specific aim within this thesis addresses these questions.

1.8. Endocannabinoid-mediated retrograde signaling

As suggested by electrophysiological studies, a retrograde signaling molecule may be responsible for the 5-HT_{2A} receptor-dependent effects observed in the prefrontal cortex. Because Kv1.2 channels appear to be the target of this retrograde transmitter, and considering that the 5-HT_{2A} receptor stimulates eicosanoid release, it is likely that an eicosanoid facilitates this response. Endocannabinoids are a class of eicosanoids that play a definite role in retrograde signaling throughout the CNS.

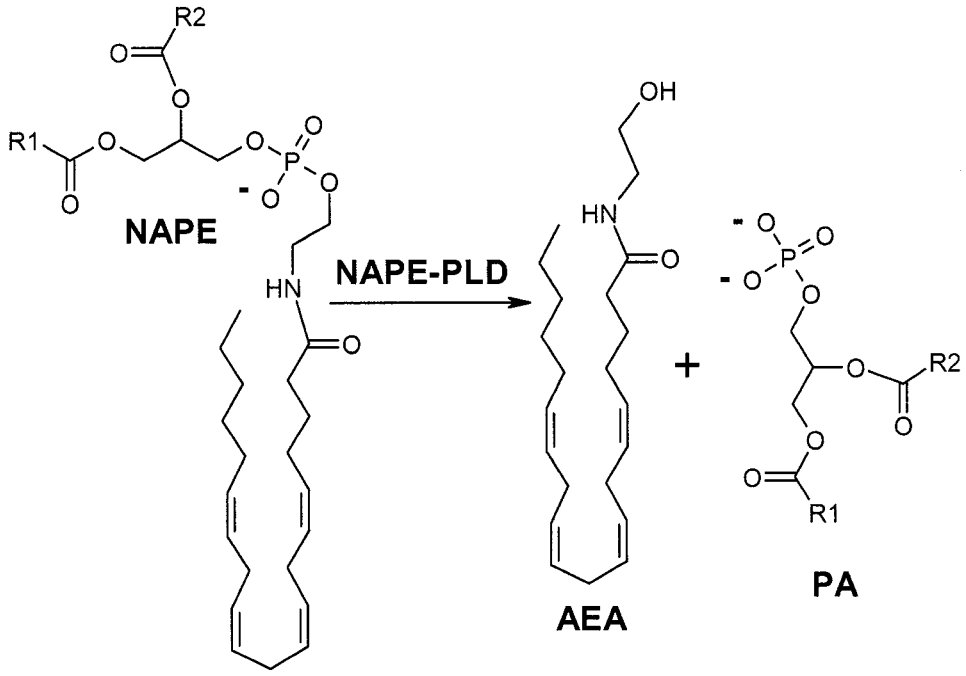


Figure 1.9 The biosynthesis of anandamide (AEA). NAPE-PLD catalyzes the hydrolysis of the phospholipid NAPE yielding AEA and PA.

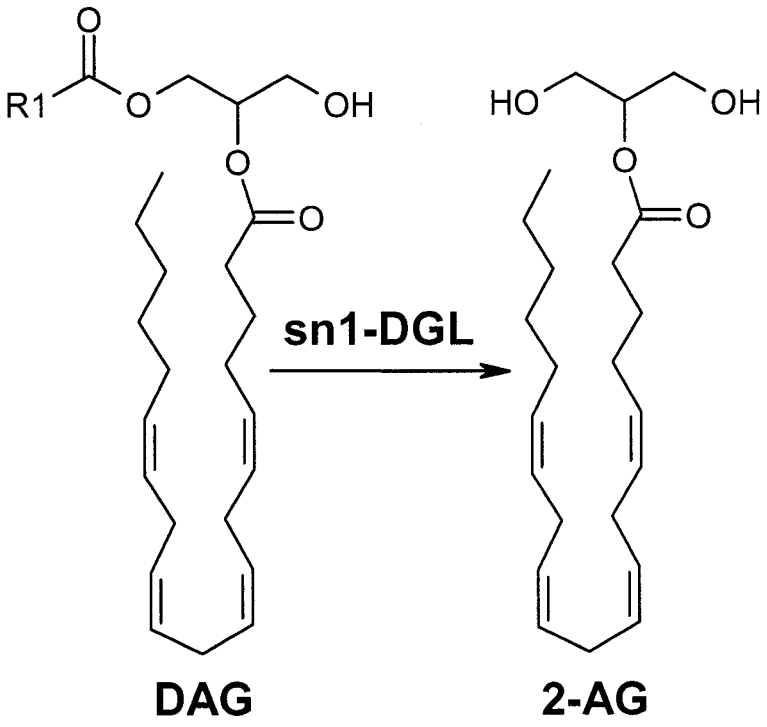


Figure 1.10 The biosynthesis of 2-arachidonoylglycerol (2-AG). The production of 2-AG is catalyzed by an sn1-specific DGL yielding 2-AG from membrane DAG.

The two main endocannabinoids discovered to date are anandamide (*N*-arachidonoylethanolamide) and 2-AG (2-arachidonoylglycerol). The direct lipid precursor of anandamide, N-arachidonoyl phosphatidylethanolamine (NAPE), is created by the condensation of arachidonic acid and phosphatidylethanolamine (PE), catalyzed by an uncharacterized N-acyltransferase (NAT). NAPE is the substrate for NAPE-specific PLD that catalyzes the cleavage of NAPE into PA and anandamide (Di Marzo *et al.* 1994;Sugiura *et al.* 1996a;Sugiura *et al.* 1996b) (Fig. 1.9). Both enzymatic reactions require increases in intracellular calcium concentration that may originate from plasma membrane ion channels or intracellular calcium stores. The endocannabinoid 2-AG is also generated in a calcium dependent way (Kondo *et al.* 1998) following the deacylation of DAG, a reaction catalyzed by an sn1-specific DAG lipase (Bisogno *et al.* 2003) (Fig. 1.10).

Both 2-AG and anandamide are implicated in depolarization-induced suppression of inhibition (DSI) as well as depolarization-induced suppression of excitation (DSE). DSI is a calcium-dependent slow type of retrograde signaling observed in numerous brain structures, including the hippocampus (Pitler and Alger 1992), the cerebellum (Llano *et al.* 1991), and the cortex (Trettel and Levine 2003). In a DSI event, increases in the intracellular calcium of a postsynaptic neuron cause the release of a retrograde messenger that diffuses to a presynaptic GABA neuron where it decreases the probability of GABA release

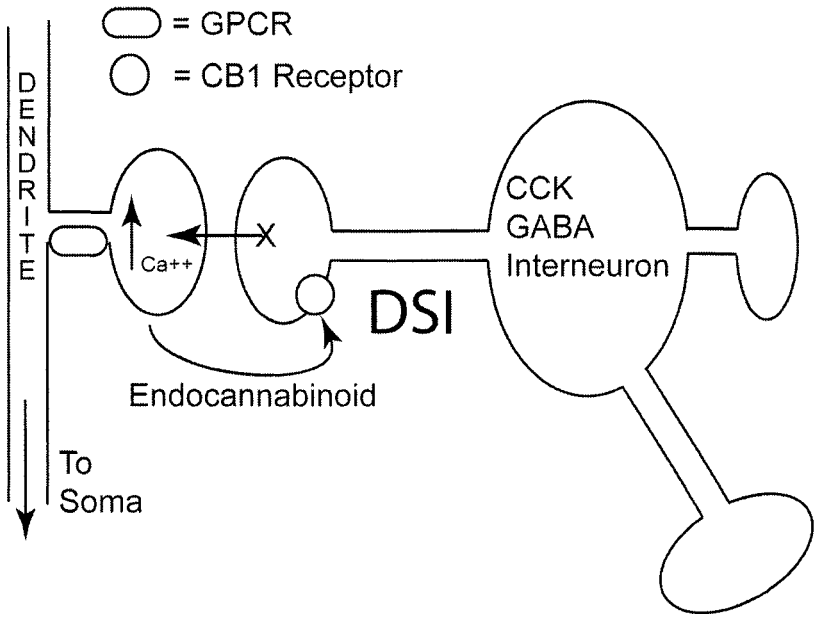


Figure 1.11 Endocannabinoid-mediated DSI. Increased calcium concentrations within the postsynaptic neuron lead to endocannabinoid production, retrograde transport, and CB1 receptor-dependent inhibition of GABA release. Calcium concentration increases may be the result of postsynaptic depolarization, GPCR activation, or both.

(i.e. inhibition of inhibition) (Alger and Pitler 1995). DSI was also found to be pertussis toxin sensitive, suggesting the involvement of $G_{i/o}$ type G-proteins (Pitler and Alger 1994). The discovery that endocannabinoids carry the retrograde message responsible for DSI provided the missing link in the mechanism of action (Ohno-Shosaku *et al.* 2001;Wilson and Nicoll 2001;Kreitzer and Regehr 2001b). In summary, increased calcium concentrations within postsynaptic neurons lead to the release of endocannabinoids, which bind to and activate presynaptic $G_{i/o}$-coupled cannabinoid receptors, leading to the inhibition of GABA release in an activity-dependent manner (Fig. 1.11).

DSI has been most extensively studied in the cerebellum and hippocampus, although recently it has been demonstrated to occur in the cortex (Trettel and Levine 2003). Anatomical studies confirm the presynaptic localization of CB1 receptors to the axon terminals of GABAergic interneurons in the rat somatosensory cortex (Bodor *et al.* 2005) and axon terminals of cortical glutamatergic neurons in mice (Domenici *et al.* 2006). Microdialysis experiments with the cannabinoid receptor 1 (CB1) agonist WIN55212-2 suggest that activation of presynaptic CB1 receptors on GABAergic interneurons in the rat cortex leads to an inhibition of GABA release (Ferraro *et al.* 2001). Additionally, electrophysiological experiments demonstrate that WIN55212-2 also inhibits glutamate release in the rat cortex (Auclair *et al.* 2000). The results of these

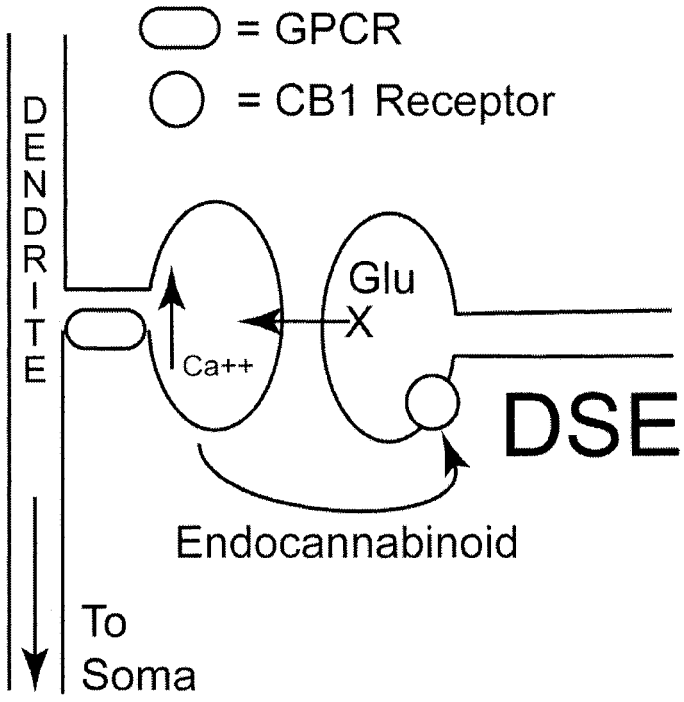


Figure 1.12 Endocannabinoid-mediated DSE. Increased calcium concentrations within the postsynaptic neuron lead to endocannabinoid production, retrograde transport, and CB1 receptor-dependent inhibition of glutamate release. Calcium concentration increases may be the result of postsynaptic depolarization, GPCR activation, or both.

studies support the general theme of presynaptic modulation of both GABA and glutamate release by agonist action at CB1 receptors in the cortex.

Depolarization-induced suppression of excitation (DSE) (Fig. 1.12) is also a calcium-dependent slow type of retrograde signaling that occurs in response to endocannabinoids and is observed in many brain regions, including the cerebellum (Kreitzer and Regehr 2001a), hippocampus, (Misner and Sullivan 1999), striatum (Gerdeman *et al.* 2002), and cortex (Auclair *et al.* 2000). In a DSE event, increases in the intracellular calcium of a postsynaptic neuron cause the release of a retrograde messenger that diffuses to a presynaptic glutamatergic neuron where it decreases the probability of glutamate release. Similar to DSI, DSE is also pertussis toxin sensitive, suggesting the involvement of $G_{i/o}$ type G-proteins (Maejima *et al.* 2001b). DSE may be of more relevance to the hallucinogen mechanism of action in the cortex as most of the studies conducted with hallucinogens support a 5-HT_{2A} receptor-mediated neuromodulatory effect on presynaptic glutamate release.

In addition to GABAergic and glutamatergic neurons, at least three neuromodulatory neurotransmitter systems in the cortex are affected by cannabinoids. Norepinephrine (NE) (Trendelenburg *et al.* 2000), 5-HT (Nakazi *et al.* 2000), and acetylcholine (Gifford and Ashby, Jr. 1996) release are all inhibited by application of CB1 receptor agonists and this effect is blocked with CB1 receptor antagonists. These effects are likely mediated by CB1 receptors located on axon terminals of the neuromodulatory afferents, although the source

of the endogenous endocannabinoids has not yet been discovered. Perhaps the endocannabinoids originate from adjacent pyramidal neurons.

In summary, the general mechanism of endocannabinoid action involves postsynaptic synthesis, retrograde transport across the synapse, and inhibition of presynaptic neurotransmitter release by agonist action on presynaptic CB1 receptors. Unlike anandamide, the endocannabinoid 2-AG is a full agonist for the CB1 receptor-dependent stimulation of $G_{i/o}$ heterotrimeric G-proteins (Savinainen *et al.* 2001) and has been found to be present in much higher concentrations in the brain relative to anandamide (Valenti *et al.* 2004). Similar to 5-HT and norepinephrine, brain concentrations of anandamide and 2-AG follow a diurnal pattern, being five-fold higher during the day for 2-AG, whereas concentrations of anandamide are noticeably higher at night (Valenti *et al.* 2004;Murillo-Rodriguez *et al.* 2006). This diurnal variation is consistent with the hypothesis that 2-AG may be involved with the neuromodulatory maintenance of the waking conscious state (Gottesmann 2002).

1.9. Endocannabinoids in the cortex

In prefrontal cortical slices, CB1 agonists have been demonstrated to cause a decrease in the frequency of EPSCs induced by electrical stimulation of layer V afferents. Curiously, inhibition of CB1 receptors with SR141716A alone was shown to cause a slight increase in EPSCs (Auclair *et al.* 2000) similar to

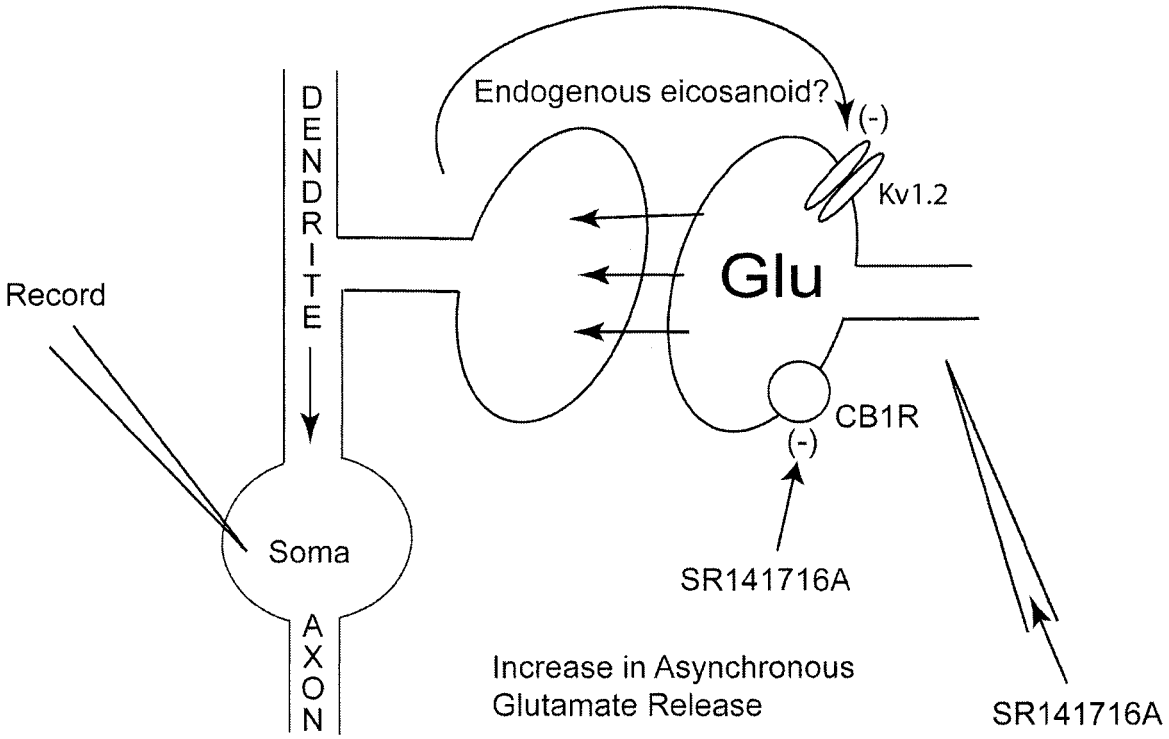


Figure 1.13 SR141716A stimulates asynchronous glutamate release. When the CB1 receptor is inhibited with SR141716A, endogenous eicosanoids may block Kv1.2 channels leading to the observed asynchronous glutamate release from glutamatergic afferents in the prefrontal cortex.

the EPSCs observed following the application of 5-HT_{2A} agonists (Aghajanian and Marek 1997;Aghajanian and Marek 1998;Aghajanian and Marek 1999;Lambe *et al.* 2000). An explanation for this observation (Fig. 1.13) that considers the results of Lambe and Aghajanian (2001), is that basal levels of endocannabinoids or possibly other endogenous eicosanoids present in the slice preparation may have been blocking Kv1.2 channels, while CB1 receptors were blocked with SR141716A, thus leading to the observed increase in glutamate release and postsynaptic EPSCs. Both CB1 receptors (Auclair *et al.* 2000;Domenici *et al.* 2006) and Kv1.2 channels (Lambe and Aghajanian 2001) are found on glutamatergic axon terminals in the cortex. Because activation of CB1 receptors leads to an inhibition of glutamate release, and blocking Kv1.2 channels leads to an increase in glutamate release, endocannabinoids with or without concurrent eicosanoid release and/or CB1 receptor blockade would be expected to have mixed effects depending on relative synaptic concentration, expression of endocannabinoid regulated proteins, and the relative affinities of endocannabinoids for the target proteins.

Serotonin and other neuromodulatory neurotransmitters may alter depolarization/calcium-induced endocannabinoid levels by either additively or synergistically enhancing endocannabinoid biosynthesis or, alternatively, by decreasing endocannabinoid uptake and hydrolysis (Fig. 1.14). Synergistic endocannabinoid release has already been demonstrated with depolarization

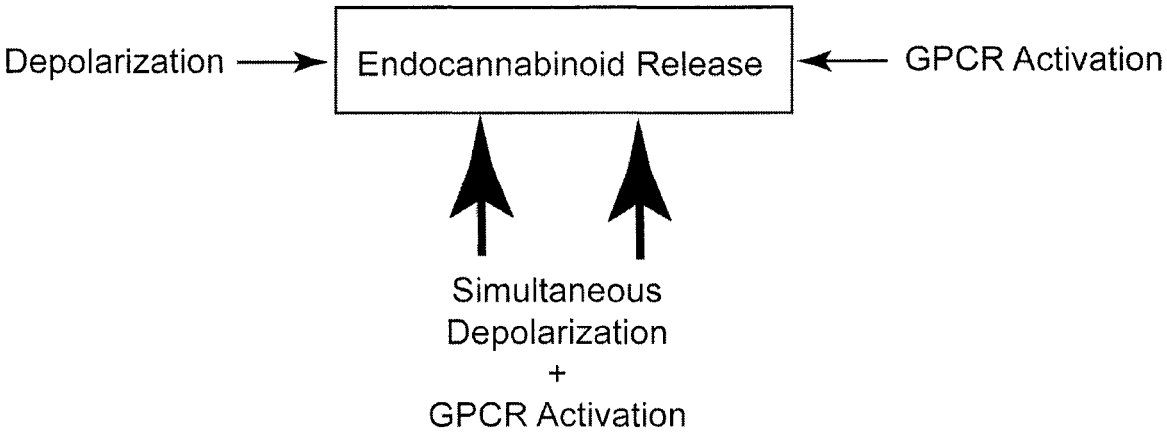


Figure 1.14 Effect of simultaneous depolarization and GPCR activation on endocannabinoid release. Whereas both depolarization and $G_{q/11}$-coupled GPCR activation can lead to endocannabinoid release, some researchers have observed an enhanced response when both stimuli are presented (Varma *et al.* 2001;Kim *et al.* 2002;Ohno-Shosaku *et al.* 2002;Ohno-Shosaku *et al.* 2003;Maejima *et al.* 2005).

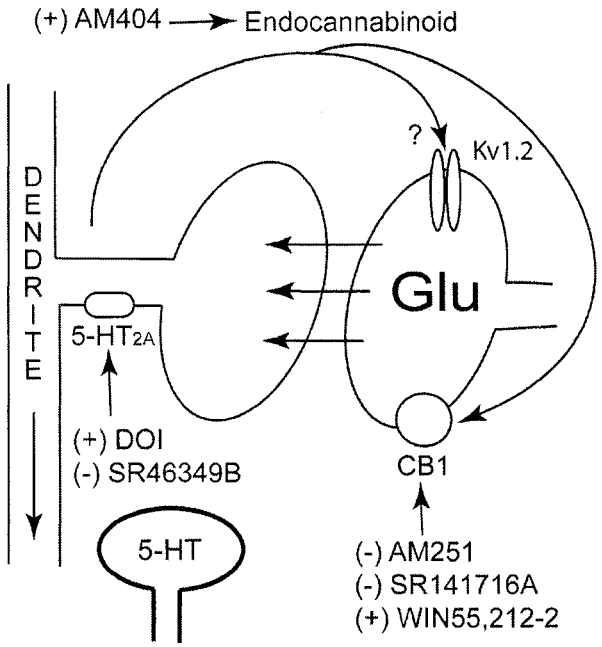
and simultaneous $G_{q/11}$-coupled mGluR1 receptor activation in the cerebellum (Maejima *et al.* 2005), the hippocampus (Varma *et al.* 2001;Ohno-Shosaku *et al.* 2002), and with $G_{q/11}$-coupled muscarinic receptors in the hippocampus (Kim *et al.* 2002;Ohno-Shosaku *et al.* 2003). It is likely only a matter of time until other $G_{q/11}$-coupled receptors are found to mediate the same effects.

1.10. Evidence for endocannabinoid involvement in 5-HT_{2A} receptor behavioral models

Animal models support a role for endocannabinoids in behaviors that result from 5-HT_{2A} receptor activation. In rats given the 5-$HT_{2A/2C}$ receptor agonist DOI, a behavioral effect known as wet dog shakes (WDS), is observed (Gorzalka *et al.* 2005). Likewise in mice, 5-HT_{2A} receptor activation reliably produces both the head-twitch response (HTR) and the ear scratch response (ESR) (Darmani 2001). Gorzalka et al., (2005) found that rats pretreated with 10 mg/kg of the endocannabinoid uptake inhibitor AM404 displayed reduced WDS following administration of DOI. This study supports the hypothesis that increased synaptic levels of endocannabinoids negatively regulate the activity of the 5-HT_{2A} receptor. In another study, mice pretreated with CB1 receptor agonists displayed reduced HTR and ESR compared to control animals when DOI was subsequently administered (Darmani 2001). This study supports the hypothesis that the CB1 receptor is the target for the endocannabinoids that negatively regulate the activity of the 5-HT_{2A} receptor. The CB1 receptor antagonist SR141716A alone increased the frequency of both HTR and ESR

(Darmani and Pandya 2000), further supporting the hypothesis that the CB1 receptor is involved in the behavioral consequences attributed to 5-HT_{2A} receptor activation. It would be prudent to remind the reader that in electrophysiological studies of layer V prefrontal cortical neurons, SR141716A (the CB1 antagonist) caused an increase in EPSCs measured in postsynaptic pyramidal neurons (Auclair *et al.* 2000) similar to what is observed when hallucinogens are applied to this area (Aghajanian and Marek 1999), suggesting a similar mechanism of action.

If we assume that the activation of 5-HT_{2A} receptors at cortical glutamatergic synapses with postsynaptic 5-HT_{2A} receptors and presynaptic CB1 receptors leads to endocannabinoid production, retrograde transport, and presynaptic CB1 receptor activation, then we would predict that this process would provide negative feedback regulation of the stimulatory activity of the 5-HT_{2A} receptor. Considering this model and the experiments mentioned above, the presence of an endocannabinoid transport inhibitor (Gorzalka *et al.* 2005) inhibits the uptake of endocannabinoids produced following 5-HT_{2A} receptor activation, leading to greater activation of CB1 receptors and inhibition of the DOI-induced WDS. The same effect explains the observations seen with the pre-administration of a CB1 receptor agonist (Darmani 2001). If this model is correct, pre-administration of a CB1 receptor antagonist would remove the hypothesized negative feedback regulation of 5-HT_{2A} receptor activity and lead to



DOI	AM251	AM404	WIN55,212-2	SR141716A	SR46349B	WDS, ESR, HTR?
X						Standard
X	X					Enhanced
X		X				Reduced
X			X			Reduced
				X		Standard
				X	X	Reduced

Figure 1.15 Summary of animal studies. On the figure of a cortical glutamatergic synapse above the table, drugs used in the animal studies are preceded by a (+) or (-) sign indicating whether they activate or inhibit respectively the receptor in question or in the case of AM404, endocannabinoid levels. The table below the figure summarizes the drug treatments with the WDS, HTR, and ESR responses observed for each treatment marked with Xs. Standard indicates a normal DOI-mediated response whereas reduced or enhanced indicate responses that were lesser or greater than the DOI response respectively.

enhanced WDS. Indeed, Gorzalka *et al.* (2005) found that preadministration of the CB1 receptor antagonist AM251 enhanced DOI-induced WDS in rats, further confirming the model. A schematic illustrating these proposed functional relationships is shown above (Fig. 1.15).

Based on results from animal studies, it is likely that endocannabinoids play a role in the mechanism of action for hallucinogens that activate the 5-HT_{2A} receptor. Whether or not an endocannabinoid is the unidentified retrograde messenger hypothesized to exist, based on electrophysiological studies, remains unknown. As will be demonstrated under the results section of specific aim 2, stimulation of the 5-HT_{2A} receptor leads to the production of the endocannabinoid 2-AG in a PLC-dependent manner.

CHAPTER 2. SPECIFIC AIMS

2.1. Rationale

The degree to which 5-HT_{2A} receptor-mediated effector pathway activation is correlated to the hallucinogenic properties of different 5-HT_{2A} receptor agonists has never been determined. One of the major problems that must be faced to solve this problem is that the 5-HT_{2A} receptor appears to couple to multiple signaling pathways, forming a complex signaling network. In NIH3T3-5HT_{2A} cells, part of this signaling web was found to be responsible for the release of eicosanoids (Kurrasch-Orbaugh *et al.* 2003a), although specific eicosanoids were never identified and total eicosanoid release was perhaps presumed to be equated to AA release resulting from PLA_2 activation. Although a direct correlation was not observed in the data presented in that study, hallucinogens tended to promote eicosanoid release over IP accumulation, as determined by EC50s and intrinsic activities, compared with nonhallucinogenic agonists. For example, the relatively potent tryptamine hallucinogens psilocin and 5-MeODMT were both much more effective agonists for stimulation of the eicosanoid release pathway relative to the PLC pathway, whereas the non-hallucinogenic unsubstituted tryptamine favored the PLC pathway over eicosanoid release. Determining which of these pathways is correlated with hallucinogenesis, if any,

would theoretically allow the development of signaling-selective drugs with possible cognitive enhancing effects and reduced or abolished hallucinogenic side effects. The thesis project described herein proposes and tests several hypotheses related to 5-HT_{2A} receptor-dependent signal transduction.

2.2. Hypotheses

2.2.1. Specific aim 1

To test the hypothesis that alpha-methyl-phenylalkylamine 5-HT_{2A} receptor agonists are more efficacious for the 5-HT_{2A} receptor-coupled PLC pathway relative to their two-carbon side chain analogues. Differential stimulation of the 5-HT_{2A} receptor-dependent PLC signaling pathway has been reported for 5-HT_{2A} receptor agonists (Berg *et al.* 1998a;Berg *et al.* 1998b;Kurrasch-Orbaugh *et al.* 2003b). A series of phenethylamines with small structural differences in their ethylamine side chains was tested to obtain a profile of PLC activation, with attention paid to relative hallucinogenic potency. As some limited human data for many of the compounds tested has been reported, functional differences compared to the effective dose required to elicit hallucinogenesis will be discussed.

2.2.2. Specific aim 2

To test the hypothesis that 5-HT_{2A} receptor stimulation leads to the release of endocannabinoids. Experiments were performed in order to establish whether 5-HT_{2A} receptor stimulation leads to endocannabinoid production in heterologous expression systems. A new downstream branch of the 5-HT_{2A} receptor-dependent PLC pathway that leads to the production of the endocannabinoid 2-AG was discovered. This branch was originally predicted to exist based on literature reports concerning the effects of hallucinogen action at glutamatergic synapses in the prefrontal cortex. More recent animal studies lend support to the findings described herein and will be discussed accordingly.

2.2.3. Specific aim 3

To test the hypothesis that 5-HT_{2A} receptor-dependent arachidonic acid release is dependent on the 2-AG production pathway. Previous studies in NIH3T3-5HT_{2A} cells have determined that 5-HT_{2A} receptor-dependent eicosanoid release is independent of PLC activation. In light of the fact that the eicosanoid 2-AG was produced in a PLC-dependent fashion, a more refined method of analyzing AA release was used to explore its possible biosynthetic routes.

2.3. Significance

The 5-HT_{2A} receptor has been implicated both as an essential component of the hallucinogen mechanism of action and as a possible factor in various CNS pathologies such as schizophrenia (Meltzer 1995;Meltzer 1999). The 5-HT_{2A} receptor also appears to be involved with working memory (Vollenweider *et al.* 1998;Williams *et al.* 2002). Since the clinical discovery that the 5-HT_{2A} receptor was critical for the subjective effects of hallucinogens in humans (Vollenweider *et al.* 1998), multiple effector pathways have been discovered, including PLC (Conn and Sanders-Bush 1984;Conn and Sanders-Bush 1985;Conn and Sanders-Bush 1986), ARF1/6 (Robertson *et al.* 2003), PLA_2 (Felder *et al.* 1990), MAPK, and RhoA (Kurrasch-Orbaugh *et al.* 2003a). It is not currently understood how the relative activation of these pathways by different 5-HT_{2A} receptor agonists may contribute to their overall effects on consciousness. A better understanding of agonist-directed trafficking (ADT) or the differential stimulation of 5-HT_{2A} receptor-dependent effector pathways by different agonists may help to explain why some 5-HT_{2A} receptor agonists are hallucinogenic whereas others are not.

CHAPTER 3. MATERIALS AND METHODS

3.1. Chemicals and supplies

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic Acid was obtained from Amersham Life Sciences (Piscataway, NJ). *Myo*-[2-^{3}H(N)]-inositol was obtained from New England Nuclear (Boston, MA). Dulbecco's Modified Eagle Medium (DMEM), G-418, and BSA were purchased from Sigma Chemical (St. Louis, MO). Dialyzed fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). A23187, Et-18-OCH_3, PMA, RHC80267, Staurosporine, and U-71322 were obtained from Calbiochem (San Diego, CA). Ketanserin and 5-HT were purchased from Research Biochemicals, Inc. (Natick, MA). Anandamide, AA, 2-AG, AM404, MAFP, and URB597 were purchased from Cayman Chemical Co. Inc. (Ann Arbor, MI). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). All of the 5-HT_{2A} receptor agonists as well as URB602 were synthesized in our laboratory. All other cell culture reagents not specifically mentioned were purchased from Gibco BRL (Carlsbad, CA)

The phospho-MAPK antibody sampler was purchased from Cell Signaling Technology (Beverly, MA). The sampler includes, in part, phospho-ERK1,2 and phospho-p38 primary polyclonal antibodies. Anti-rabbit, Ig, fluorescein-linked whole antibody and anti-fluorescein alkaline phosphatase conjugate were

purchased from Amersham Life Sciences (Piscataway, NJ) and employed as secondary and tertiary antibodies, respectively.

3.2. Cell culture and transfections

The NIH3T3-5HT$_{2A}$ and NIH3T3-5HT$_{2C}$ cell lines stably expressing the rat 5-HT$_{2A}$ receptor (5500 fmol/mg) or the rat 5-HT$_{2C}$ receptor (7500 fmol/mg), respectively, were the kind gift of Dr. David Julius of the Department of Pharmacology, University of California, 513 Parnassus St., San Francisco, CA 94143. CHO cells stably expressing the human 5-HT$_{1A}$ receptor (500 fmol/mg) were the kind gift of Christopher Harber. A549 cells, stably expressing the human 5-HT$_{2A}$ receptor (150 fmol/mg protein) (A20 cells) were the kind gift of Dr. Ulrike Weyer-Czernilofsky of the Ernst Boehringer Institute, Bender and Co., Vienna Austria. NIH3T3-5HT$_{2A}$, NIH3T3-5HT$_{2C}$, and A20 cells were maintained in culture flasks at 37 °C with 5% CO_2 in DMEM supplemented with 10% (v/v) dialyzed FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 300 mg/L G-418. CHO-1A cells were maintained in culture flasks at 37 °C with 5% CO_2 in DMEM supplemented with 10% (v/v) dialyzed FBS, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 22.5 units of hygromycin B/L. All cells were passaged at 90-95% confluency and discarded after approximately 30 passages or when they failed to respond to 5-HT.

3.3. Receptor binding assays

3.3.1. Preparation of membranes

NIH3T3-5HT$_{2A}$, NIH3T3-5HT$_{2C}$, CHO-1A, and A20 cells were maintained in 150 mm tissue culture dishes in standard growth media, as described above, until approximately 90% confluent. Prior to preparing membranes, the media was removed by aspiration, cells were rinsed with phosphate-buffered saline (PBS), and the media was replaced with unsupplemented serum-free Opti-MEM. After five hours the plates were placed on ice for ten minutes, cells were scraped into 50 mL centrifuge tubes, and the tubes were centrifuged at 2000 x g (4 °C) for ten minutes. Following aspiration of the supernatant, the resulting pellet was resuspended in ice-cold PBS and transferred in 1 mL aliquots into microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 15000 x g (4 °C) for 20 minutes. Following the second centrifugation, the supernatant was removed by aspiration and the resulting pellet was placed in a freezer at -80 °C until needed.

3.3.2. Saturation binding assay

Saturation binding assays were performed with 0.313 to 10 nM [^{3}H]-ketanserin, or 0.313 to 10 nM [^{3}H]-mesulergine, for the 5-HT$_{2A}$ or 5-HT$_{2C}$ receptor, respectively. In some experiments, 0.125 to 5 nM of the agonist radioligand [^{125}I]-DOI was used for either receptor. In experiments with the 5-

HT_{1A} receptor, 0.25 to 10 nM [^{3}H]-8-OH-DPAT was used. Nonspecific binding was defined as that measured in the presence of 10 μM cinanserin, 10 μM mianserin, or 10 μM 5-HT, for the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{1A} receptors, respectively. All drugs and radioligands were diluted in binding buffer (50 mM Tris, 0.5 mM EDTA, 10 mM $MgCl_2$; pH = 7.4). To each well of 96-well test tube racks were added 200 μL of binding buffer and 25 μL of radioligand. In wells representing the nonspecific binding, the binding buffer was reduced to 175 μL, and 25 μL of cinanserin, mianserin, or 5-HT was added, as noted above. The assay was initiated by the addition of 25 μL (25 mg) of cellular homogenate to each well. Following 60 minute incubation at room temperature, the reaction was terminated by rapid filtration through GF-B Uni-filters pretreated with 0.3% polyethylenimine using a pre-chilled Packard 96-well harvester. The tubes were washed three times with 4 °C wash buffer (10 mM Tris, 150 mM NaCl) and filter plates were left to air dry overnight. The following day, 40 μL of Microscint-O was added to each well and radioactivity was assessed with a TopCount (Packard) scintillation counter. Data were analyzed and saturation curves were generated using GraphPad Prism software.

3.3.3. Competition binding assay

Competition binding assays were conducted using the same equipment and methodology as the saturation binding assay, but with the following changes. The total volume per well was increased to 500 μL and a single concentration of

radioligand was utilized. In the case of [^{3}H]-ketanserin, [^{3}H]-mesulergine, and [^{3}H]-8-OH-DPAT, a 1 nM concentration was used. For [^{125}I]-DOI, a 0.2 nM concentration was used. Serial dilutions of test compounds were added to each well and the reaction was initiated by the addition of 50 μL (50 mg) of cellular homogenate to each well. The methods were as described above. Data were analyzed and competition binding curves were generated using GraphPad Prism software.

3.4. Lipid metabolite assays

3.4.1. PI hydrolysis assay

Accumulation of total IP was determined using a modified version of a previously published protocol (Berg *et al.* 1994). Cells were seeded in 48-well plates for a final density of 1 x 10^5 cells/well in standard growth media prepared as described above. After the final desired cell density was reached, the media was replaced with serum- and inositol-free CRML-1066 media, supplemented with 1.0 μCi/mL *myo*-[2-^{3}H(N)]-inositol, and incubation was allowed to proceed for 18 hours. Prior to the assay, the cells were pretreated for 15 minutes at 37 °C with 10 μM pargyline, 10 mM LiCl, and any inhibitors. Following this incubation, the cells were then stimulated with agonists for 30 minutes at 37 °C. The assay was terminated by aspiration of the medium and the addition of 10 mM formic acid (300 μL/well). After a 24 hour incubation at 4 °C, the [^{3}H]-phosphoinositides

were separated as follows. A 250 μL aliquot from each well was placed onto a small ion exchange column that had been pre-equilibrated with equilibrium buffer (10 mM myo-inositol, 3 M ammonium formate; 1 x 15 mL per column) (Berridge 1983). Columns were 9 x 60 mm, each containing 1 g of Dowex-1 ion exchange resin, which filled the column to a depth of approximately 20 mm. Each column was then rinsed by gravity elution of 15 mL of equilibrium buffer. Next, the columns were each washed with 15 mL of wash buffer (5 mM sodium tetraborate, 10 mM ammonium formate) and again allowed to drain by gravitational flow. After placing the columns over plastic scintillation vials, the [^{3}H] inositol phosphates were eluted from the columns with 2.0 mL of elution buffer (1.0 M ammonium formate and 0.10 M formic acid). Eco-lite scintillation cocktail was added (15 mL) to each vial and the radioactivity was quantified using a Beckman LS6500 liquid scintillation counter. The columns were rejuvenated by washing once with 2.5 mL rejuvenation buffer (0.10 M formic acid, 3 M ammonium formate), and re-equilibrated with 15 mL of equilibrium buffer. Using GraphPad Prism software, data were normalized to basal and 10 μM 5-HT responses and variable slope dose-response curves were generated for agonists. Average EC50s and intrinsic activities with SEMs were calculated for at least three independent experiments.

3.4.2. Eicosanoid and endocannabinoid release assays

The quantity of total released eicosanoids was determined using a modified version of the procedure of Berg *et al.* (1998b). Cells were seeded in 24-well plates at a density of 2 x 10^5 cells/well in standard growth media and incubated until they reached approximately 70% confluency. After the desired final cell density was reached, the media was aspirated and replaced with 500 μL/well full growth media, supplemented with 0.5 μCi/mL [^{3}H]AA and 10 μM unlabeled AA. After five hours incubation, the media was aspirated and the plates were washed for 15 minutes at 37 °C by replacing the media with serum-free DMEM supplemented with 0.5% fatty acid-free BSA. Inhibitors or antagonists, if necessary, were present during the wash. Agonist was added and the assay was allowed to incubate for 30 minutes at 37 °C. Following this final incubation, a 100 μL aliquot of the cell medium was removed and added to scintillation vials containing 5 mL Eco-lite scintillation cocktail. The radioactivity (total eicosanoids) was quantified using a Beckman LS6500 liquid scintillation counter. Using GraphPad Prism software, data were normalized to basal and 10 μM 5-HT responses and variable slope dose-response curves were generated for agonists. Average EC50s and intrinsic activities with SEMs were calculated for at least three independent experiments.

The quantity of AA and 2-AG released was determined using a modified version of the procedure for eicosanoid release described above. Each treatment condition represented four wells of a 24-well plate. Inhibitors or antagonists were present during both this wash and the subsequent incubation

with the agonist. When attempting to obtain 2-AG release dose response curves for agonists, 500 nM URB597 was included in the assay to inhibit 2-AG hydrolysis. Following removal of the wash media and addition of fresh serum-free DMEM supplemented with 0.5% fatty acid-free BSA and reapplication of antagonists and inhibitors when necessary, the assay was initiated by the addition of 5-HT (10 μM), followed by incubation for 10 minutes at 37 °C. The total volume of media per well at this stage was 500 μL, including 5-HT and any antagonists or inhibitors. After this final incubation, 50 μL aliquots of the cell medium were removed from each well, added to scintillation vials, and quantified using a Beckman LS6500 liquid scintillation counter. These data represented the total [^{3}H]AA-labeled eicosaniods released by the cells.

After removal of this aliquot, the balance of the media (1.8 mL/treatment pooled from 4 wells) was removed and added to a 15 mL centrifuge tube kept on ice that contained 3.2 mL $CHCl_3$, 0.8 mL MeOH, and 80 μL of 1 M HCl (40:10:1). The phases were mixed twice for 5 seconds with a vortex shaker and then separated by centrifugation (2000 x g, 15 min, 0 °C). The $CHCl_3$ layer was removed from each sample and evaporated under a stream of argon. These sample concentrates were resuspended in 52 μL $CHCl_3$ and spotted onto 20 x 20 cm glass-backed thin layer silica gel chromatography (TLC) plates (two 20 μL spots/sample). The TLC plates were developed using an 13:5 ethyl acetate:isooctane mobile phase saturated with 10% (v/v) acetic acid. After drying the developed plates, the lipids were visualized with iodine vapor.

Locations of the AA and 2-AG spots were identified by comparison with authentic standards. The spots were then scraped from the plates into scintillation vials, allowed to equilibrate for three days in scintillation fluid, and radioactivity was quantified with a Beckman LS6500 liquid scintillation counter. Using GraphPad Prism software, data were normalized to basal and 10 μM 5-HT responses and variable slope dose-response curves were generated for agonists. Average EC50s and intrinsic activities with SEMs were calculated for at least three independent experiments.

3.4.3. Phosphatidylbutanol accumulation assay

Cells were seeded into 6-well tissue culture plates, allowed to reach 90% confluency, and incubated for 24 hours prior to assay with 2.0 μCi/mL [^{3}H]palmitic acid in unsupplemented DMEM media. The cells were then washed by incubation for 15 minutes at 37 °C with 2 mL per well of serum-free DMEM (pH 7.4) supplemented with 0.5% fatty acid-free BSA. Following the wash step, the media was replaced with serum-free DMEM (pH 7.4) supplemented with 2 mL of 0.5% fatty acid-free BSA and 0.3% n-butanol. After 30 min treatment with agonists, cells were assayed for the accumulation of phosphatidyl butanol (PtBu). After the removal of media the plate was placed on ice and 1 mL of ice cold MeOH / 0.1 M HCl in H_2O (1:1) was added to each well. Cells were scraped and transferred to glass tubes containing 1 mL ice cold $CHCl_3$. The phases were mixed by vigorously pipetting the upper phase into the lower phase 10 times.

Upon separation, the lower phase was transferred to a fresh glass tube and the $CHCl_3$ was evaporated under a stream of argon. These sample concentrates were resuspended in 52 μL $CHCl_3$ and spotted onto 20 x 20 cm glass-backed TLC plates (two 20 μL spots/sample). The TLC plates were developed using an 11:5 Ethyl acetate:isooctane mobile phase saturated with 10% (v/v) acetic acid. After drying the developed plates, the lipids were visualized with iodine vapor. The PtBu spots were located by comparison with an authentic standard and confirmed by linear radioscan. The spots were then scraped from the plates into scintillation vials, allowed to equilibrate for three days in scintillation fluid, and radioactivity was quantified with a Beckman LS6500 liquid scintillation counter. Using GraphPad Prism software, data were normalized to basal and 10 μM 5-HT responses and variable slope dose-response curves were generated for agonists. Average EC50s and intrinsic activities with SEMs were calculated for at least three independent experiments.

3.4.4. Linear radioscan of TLC plates

Several representative TLC plates were scanned using a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer (Berthold Systems INC. Pittsburg, PA) to determine that the Rf values of radioactive peaks corresponded to authentic standards of PtBu, AA, 2-AG, and anandamide. Plates were scanned for 30-60 minutes and peaks were quantified using Chroma software (Berthold Systems INC. Pittsburg, PA).

CHAPTER 4. RESULTS AND DISCUSSION

4.1. Functional effects of the α-methyl carbon of phenylisopropylamines

In order to meet the objectives of specific aim 1, a small series of substituted phenethylamine 5-HT_{2A} agonists (Fig. 4.1) was systematically characterized for binding affinity and IP accumulation in both the cloned rat and human 5-HT_{2A} receptors. The study was initiated in order to determine whether there were any significant differences between phenethylamines and their chiral phenylisopropylamine analogues.

4.1.1. Results

The effect of the alpha methyl carbon on binding affinities at both the rat and human 5-HT_{2A} receptors

Initial studies were designed to test the hypothesis that phenethylamines and their (±)-phenylisopropylamine analogues did not significantly differ in 5-HT_{2A} receptor affinity. The binding affinity of four analogue pairs was thus determined by radioligand competition assay with [^{125}I]DOI in NIH3T3-5HT_{2A} cells expressing the rat 5-HT_{2A} receptor (5500 fmol/mg protein). The binding affinities determined for all analogue pairs were statistically indistinguishable (Table 4.1). The binding

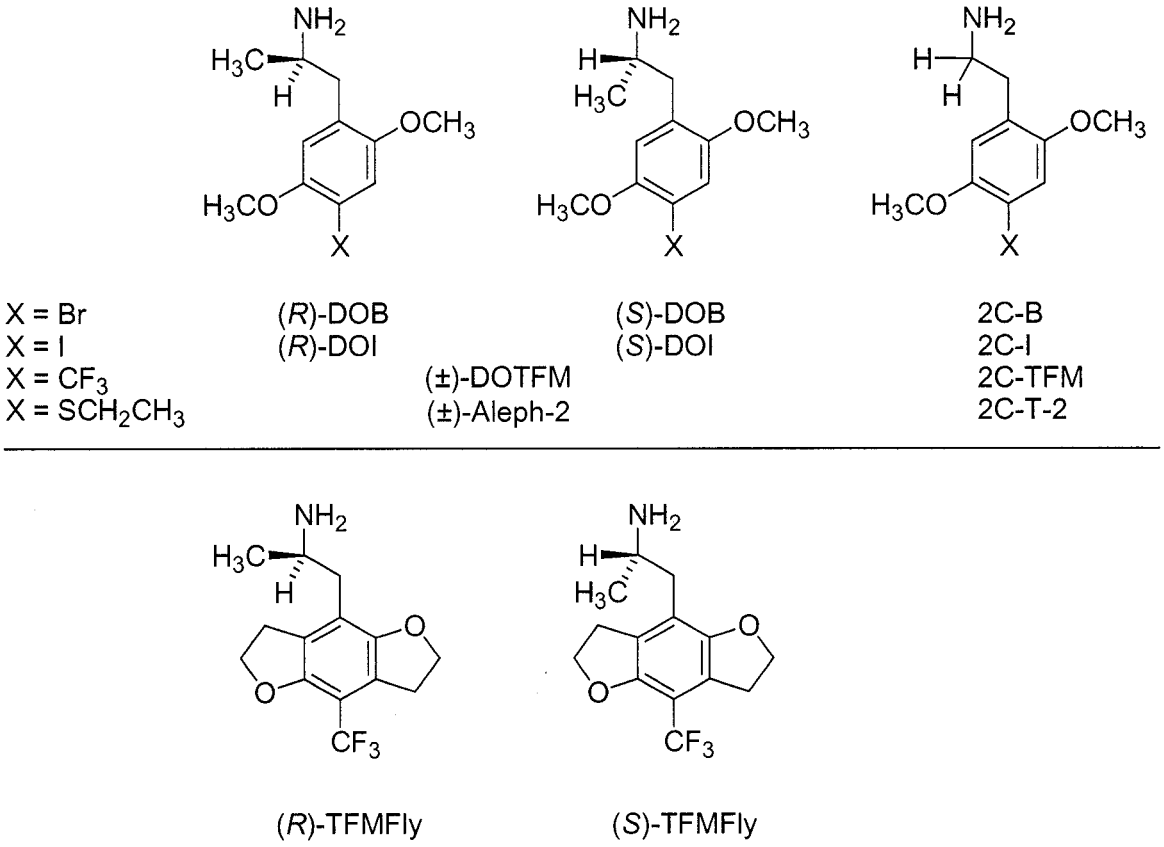


Figure 4.1 Phenethylamine and phenylisopropylamine compounds used in this study. DOTFM and Aleph-2 were not available as the pure enantiomers and were only tested as the racemic (±) materials.

affinities of two of the analogue pairs were also assessed at the human 5-HT_{2A} receptor. The affinities of the 4-iodo and 4-bromo analogue pairs proved to be statistically indistinguishable in the Hh2A cell line, expressing the human 5-HT_{2A} receptor (8000 fmol/mg protein) (Table 4.1).

Determination of the relative binding affinities for both the rat and human 5-HT_{2A} receptor of (*R*)-phenylisopropylamine and (*S*)-phenylisopropylamines with identical 4-position substituents

The binding affinities of enantiomeric phenylisopropylamine pairs were also compared at the rat and human 5-HT_{2A} receptors. The (*R*)-isomers of the four enantiomeric pairs exhibited higher affinity for the rat 5-HT_{2A} receptor relative to the affinity of the (*S*)-isomers. Additionally, the (*R*)-isomer of DOB exhibited higher affinity relative to the (*S*)-isomer at the human 5-HT_{2A} receptor (Table 4.2).

The effect of the alpha methyl carbon on PLC activation at both the rat and human 5-HT_{2A} receptors

Having shown that phenethylamines and their (±)-phenylisopropylamine analogues have indistinguishable binding affinities for both the rat and human 5-HT_{2A} receptor, we subsequently determined the relative ability of these ligands to stimulate 5-HT_{2A} receptor-dependent IP accumulation. In A20 cells, with relatively low expression levels of the human 5-HT_{2A} receptor (150 fmol/mg

Table 4.1 Comparison of affinities and potencies for PI hydrolysis of racemic PIAs their corresponding PEAs. Values are the mean and (SEM).

DRUGS	Hh2A ($h5\text{-}HT_{2A}$)	A20 ($h5\text{-}HT_{2A}$)		NIH3T3-$5HT_{2A}$ ($r5\text{-}HT_{2A}$)		
	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)
(±)-DOB	0.52 (0.08)	17.4 (3.0)	70 (4)	0.66 (0.13)	2.9 (2.1)	87 (2)
2C-B	0.69 (0.03)	18.2 (2.3)	40 (5)*	0.66 (0.11)	27.0 (2.0)	65 (5)*
(±)-DOI	0.58 (0.06)	9.7 (1.8)	50 (2)	0.65 (0.12)	19.2 (2.6)	77 (3)
2C-I	0.62 (0.08)	9.8 (1.7)	30 (2)*	0.65 (0.07)	19.0 (2.6)	59 (4)*
(±)-DOTFM	ND	10.1 (1.6)	50 (4)	0.61 (0.09)	57.0 (2.0)	78 (4)
2C-TFM	ND	7.7 (1.5)	26 (2)*	0.65 (0.10)	47.6 (5.3)	42 (3)*
(±)-Aleph-2	ND	13.1 (1.6)	57 (4)	1.78 (0.30)	76.3 (4.3)	84 (2)
2C-T2	ND	14.4 (2.1)	44 (5)	1.81 (0.23)	84.6 (12.7)	79 (2)

* $p < 0.05$ compared to the PIA analogue. ND = not determined

Table 4.2 Comparison of PIA enantiomer affinities and potencies for PI hydrolysis. Values are the mean and (SEM).

DRUGS	Hh2A ($h5\text{-}HT_{2A}$)	A20 ($h5\text{-}HT_{2A}$)		NIH3T3-5HT_{2A} ($r5\text{-}HT_{2A}$)		
	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)	K_i [^{125}I]DOI (nM)	EC50 PI hydrolysis (nM)	Intrinsic Activity (% 5-HT)
(*R*)-DOB	0.33 (0.06)	6.5 (1.2)	80 (5)	0.27 (0.05)	15.3 (2.5)	82 (1)
(*S*)-DOB	2.0 (0.2)*	25.3 (1.0)*	50 (8)*	1.49 (0.27)*	74.4 (10.5)*	76 (3)
(*R*)-DOI	ND	4.8 (0.3)	60 (2)	0.31 (0.03)	17.2 (2.9)	75 (4)
(*S*)-DOI	ND	14.7 (0.6)*	38 (1)*	0.98 (0.17)*	54.2 (7.9)*	62 (7)
(*R*)-TFMfly	ND	5.0 (1.0)	73 (5)	0.15 (0.01)	9.6 (1.1)	79 (4)
(*S*)-TFMfly	ND	8.3 (1.2)	45 (2)*	0.34 (0.05)*	8.1 (0.4)	70 (6)

*$p < 0.05$ compared to the R isomer. ND = not determined.

protein), the potency (EC50) was virtually identical for 5-HT_{2A} receptor-dependent IP accumulation stimulated by either (±)-phenylisopropylamines or their non-chiral phenethylamine analogues A significant difference in intrinsic activity was observed between each analogue pair, excluding only the 4-ethylthio analogue pair (P = 0.08) (Table 4.1). Of the 4-bromo, 4-iodo, and 4-trifluoromethyl analogue pairs, the (±)-phenylisopropylamine analogues all had higher intrinsic activity relative to their achiral phenethylamine analogues.

The ability of the four analogue pairs to stimulate PLC via the rat 5-HT_{2A} receptor expressed in NIH3T3-5HT_{2A} cells was examined. These cells have a large receptor reserve for the PLC signal transduction pathway (Kurrasch-Orbaugh *et al.* 2003b). Similar to observations with the human receptor, no difference in potency could be detected between each analogue pair for 5-HT_{2A} receptor-dependent IP accumulation, and there was a significant difference between the relative intrinsic activity of each analogue pair, again excluding only the 4-ethylthio analogue pair (P = 0.10) (Table 4.1).

Determination of the relative human 5-HT_{2A} receptor-dependent PLC activation of (*R*)- and (*S*)-phenylisopropylamines with identical 4-position substituents

After determining that the binding affinity for both the rat and human 5-HT_{2A} receptors of the (*R*)- phenylisopropylamine isomers was greater, relative to the (*S*)- phenylisopropylamine isomers, the functional properties of the isomers, as measured by IP accumulation, were determined. In A20 cells, the (*R*)-isomers of the 4-bromo and 4-iodo enantiomeric phenylisopropylamine pairs were

significantly different from the (*S*)-enantiomers, with the (*R*)-enantiomers having lower EC50s (Table 4.2). Curiously, the EC50s for the (*R*)- and (*S*)- TFMfly analogue pair were statistically indistinguishable (P = 0.08), although the relative intrinsic activities of all the enantiomeric pairs were significantly different, with the (*R*)-isomers having higher intrinsic activity compared to the (*S*)-isomers. As with the binding data, this result was consistent with *in vivo* data, where (*R*)-enantiomers are more potent than the (*S*)-enantiomers (Shulgin 1973;Shulgin and Shulgin 1991).

Determination of the relative rat 5-HT_{2A} receptor-dependent PLC activation of (*R*)- and (*S*)-phenylisopropylamines with identical 4-position substituents

The results with the rat 5-HT_{2A} receptor differed somewhat from those obtained with the human receptor. Similar to the A20 cells, the EC50s for 5-HT_{2A} receptor-dependent IP accumulation in NIH3T3-5HT_{2A} cells were significantly different for the enantiomeric pairs of the 4-bromo and 4-iodo phenylisopropylamines and the EC50s for the (*R*)- and (*S*)-TFMfly analogue pair were statistically indistinguishable (P = 0.32). The main difference between the rat and human receptors was that the intrinsic activities of all three enantiomeric pairs for IP accumulation in the NIH3T3-5HT_{2A} cells were statistically indistinguishable between analogues, whereas differences were observed in the A20 cells (Table 4.2).

4.1.2. Discussion

Contrary to the idea that the human potency differences of phenethylamines relative to their phenylisopropylamine analogues can be explained by the relative binding affinity, no differences in binding affinity between phenethylamine and (±)-phenylisopropylamine analogue pairs were observed, a result consistent with previous reports (Johnson *et al.* 1987;Nash *et al.* 1994). Based on the result of this study, however, the analogue pairs have marked differences in ability to activate the PLC second messenger system. The differential effect on a second messenger system for the 5-HT_{2A} receptor was first reported for the 4-trifluoromethyl phenethylamine and its racemic phenylisopropylamine analogue (Nichols *et al.* 1994).

The present study, using a larger series of analogue pairs, including phenylisopropylamine enantiomers, conclusively demonstrates that the phenylisopropylamines have higher intrinsic activities relative to their phenethylamine analogues for stimulating both rat and human 5-HT_{2A} receptor-dependent IP accumulation. There was no difference in either binding affinity or EC50 between any of the phenethylamine/(±)-phenylisopropylamine pairs. Only the 4-ethylthio phenethylamine/(±)-phenylisopropylamine pair lacked a significant intrinsic activity difference at either the rat or human receptor. Curiously, this result is somewhat consistent with the limited human pharmacology of this analogue pair. For example, the phenylisopropylamine analogue Aleph-2 is only two-fold more potent at inducing hallucinogenesis relative to the phenethylamine analogue, whereas both (±)-DOB and (±)-DOI are approximately ten times more

potent than their corresponding phenethylamine analogues (Shulgin and Shulgin 1991). Perhaps the more polarizable sulfur substituent in the 4-position of the aromatic nucleus allows a unique binding pose that confers higher intrinsic activity on phenethylamine partial agonists independent of the side chain alpha-methyl moiety.

Radioligand competition assays have determined that *(R)*-isomers of hallucinogenic phenylisopropylamines have higher affinity than their corresponding *(S)*-isomers (Johnson *et al.* 1990). In both the rat and human expression systems used in this study, the (*R*)-isomers of the phenylisopropylamines were consistently more potent and had higher intrinsic activities for stimulating human 5-HT_{2A} receptor-dependent IP accumulation relative to their phenethylamine analogues. In the cell line expressing the rat 5-HT_{2A} receptor, however, there was no difference in intrinsic activity between the (*R*)- and (*S*)-phenylisopropylamine analogues. This result could be a consequence of the high receptor expression levels and the large receptor reserve for PLC activation in the NIH3T3-5HT_{2A} cells (Kurrasch-Orbaugh *et al.* 2003b). Alternatively, it is possible that the observations may simply reflect subtle signaling property differences between human and rat receptors.

The lack of substantial differences in 5-HT_{2A} receptor-dependent PLC activation between the (*R*)- and (*S*)-TFMfly analogues suggests the possibility of a unique binding orientation for these benzodifuranyl ligands. The rigidity added to the phenylisopropylamine pharmacophore by the furanyl groups may yield

advantages to 5-HT_{2A} receptor interaction that outweigh the effects of side chain orientation.

The results of this study appear to agree well with the limited human pharmacology that is available for the compounds tested. In most cases the phenylisopropylamine analogues had significantly higher intrinsic activities for IP accumulation relative to their phenethylamine analogues, and (*R*)-phenylisopropylamines were significantly more potent for IP accumulation relative to the (*S*)- phenylisopropylamines. The results of this study could lead one to conclude that 5-HT_{2A} receptor-dependent IP accumulation is correlated with hallucinogenic potency. Upon looking outside of the phenethylamine world, however, the correlation ceases to exist. LSD one of the most potent hallucinogens known, yet has an intrinsic activity relative to 5-HT for IP accumulation of only 25%. Also the tryptamine hallucinogens, 5-MeODMT and psilocin, have EC50s for IP accumulation that would suggest that they are not hallucinogenic or that the human dose would have to be at least an order of magnitude higher than is actually observed in order to elicit hallucinogenic effects. Obviously, the situation is more complicated than we might believe, based on the results of this study with phenylalkylamines. The next two results sections expand our view of 5-HT_{2A} receptor signal transduction and, while not leading us to definitive answers, may lead to future experiments that may.

4.2. Serotonin 5-HT_{2A} receptor activation induces 2-AG release through a phospholipase C-dependent mechanism.

The main objectives of this study were to determine whether 5-HT_{2A} receptor activation leads to the release of endocannabinoids in a mammalian expression system, and if so, to identify the signal transduction pathways responsible for this endocannabinoid production. In the following discussion, evidence is presented that the rat 5-HT_{2A} receptor mediates the formation and release of the endocannabinoid 2-AG through a PI-PLC dependent mechanism. The 5-HT_{2A} receptor-dependent PLD pathway was found not to contribute to 2-AG release but evidence will be presented that it may play a role in 2-AG metabolism.

4.2.1. Results

The effect of 5-HT_{2A} receptor activation and the calcium ionophore, A23187 on [^{3}H]-2-AG release in NIH3T3-5HT_{2A} cells.

Experiments were performed in order to determine whether measurable quantities of endocannabinoids could be extracted from NIH3T3-5HT_{2A} cells expressing the rat 5-HT_{2A} receptor (5500 fmol/mg protein), following receptor activation. NIH3T3-5HT_{2A} cells averaged a three-fold (two- to eight-fold range) increase over basal in both [^{3}H]-2-AG and [^{3}H]-AA release following 10 minute stimulation with 10 μM 5-HT. The overall quantity of [^{3}H]-2-AG and

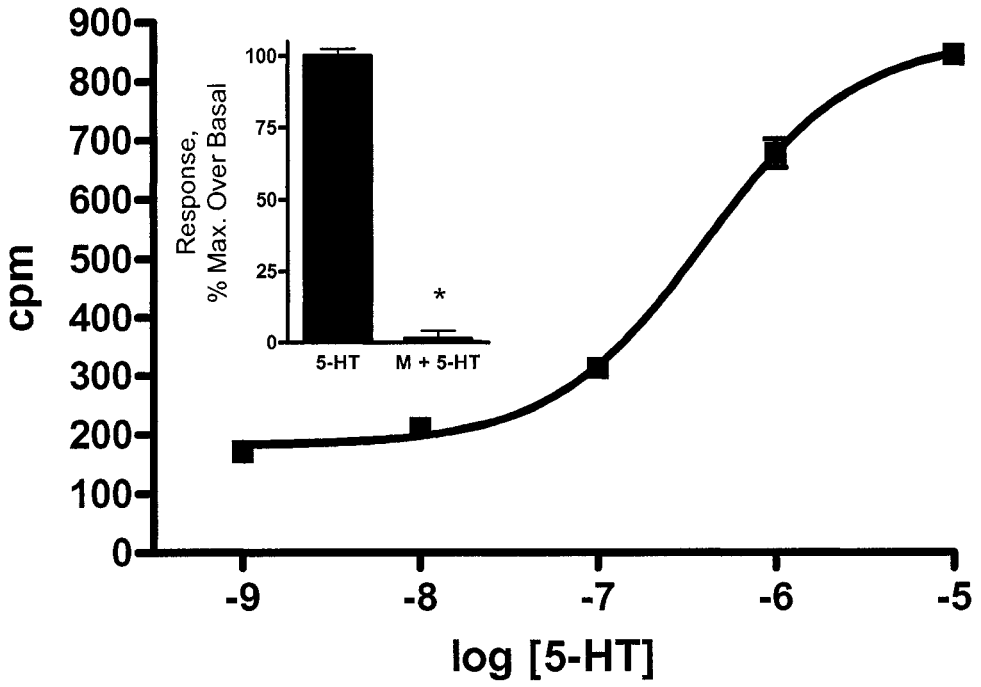


Figure 4.2 The release of 2-AG stimulated by the 5-HT_{2A} receptor is dose-dependent. NIH3T3-5-HT_{2A} cells were incubated with [^{3}H]-AA for 24 hours prior to experiments. The curve illustrates one typical experiment showing [^{3}H]-2-AG release following 10 min stimulation with serial dilutions of 5-HT. The inset graph illustrates the effect of 1 μM M100907 on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. Basal [^{3}H]-2-AG release averaged 80 ± 3 dpm and 5-HT-stimulated [^{3}H]-2-AG release averaged 220 ± 29 dpm. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. *$p < 0.05$ compared with 5-HT control.

[^{3}H]-AA was increased at longer incubation times but the fold over basal remained the same for both lipids after 30 minutes. Subsequent experiments were performed with 10 minute incubation times because after this time point the DGL inhibitor RHC80267 failed to inhibit [^{3}H]-AA release completely. Recombinant A549 and HEK cell lines expressing the human 5-HT_{2A} receptor (150 fmol/mg and 8000 fmol/mg protein, respectively) also exhibited similar increases over basal of both [^{3}H]-2-AG release and [^{3}H]-AA release following 10 min stimulation with 10 μM 5-HT (data not shown). The quantity of [^{3}H]-AA released in any given assay was approximately two times greater than that of [^{3}H]-2-AG when TLC spots from the same treatment lane were quantified. Application of 5-HT to wild type NIH3T3 cells did not result in release of [^{3}H]-2-AG, despite the report that these cells express an endogenous 5-HT_{2A} receptor (Saucier and Albert 1997).

TLC plates were scanned to verify the migration ratios of the radioactive lipids. The major peak represented, on average, approximately 37% of the gross cpm for the 5-HT lane and had an R_f (0.73) corresponding to an authentic AA standard. The second most abundant peak represented, on average, approximately 17% of the gross cpm for the 5-HT lane and had an R_f (0.43), corresponding to authentic 2-AG standard. The third peak was at the origin and represented, on average, approximately 12% of the gross cpm for the 5-HT lane. The balance of the radioactivity (about 34%) was distributed evenly along the lane and represented the background radiation. No other major peaks were observed, and a peak corresponding to anandamide was not detected. Two

additional TLC solvent systems were employed in order to confirm that the authentic standards co-migrated with peaks identified by radioscan. In diethyl ether:hexane (4:3) the R_fs of [^{3}H]-AA and [^{3}H]-2-AG were 0.34 and 0.05, respectively, and in ethyl acetate:hexane (5:2) the R_fs were 0.50 and 0.28, respectively, corresponding to authentic standards visualized with iodine vapor.

The 5-HT-stimulated release of [^{3}H]-2-AG was dose dependent (Fig. 4.2) and was completely inhibited by the selective 5-HT_{2A} receptor antagonist M100907 (Fig. 4.2 inset) (Sorensen *et al.* 1993;Kehne *et al.* 1996). The IC50 for this inhibition was determined to be 4.6 nM, with maximal inhibition occurring in the 0.5 to 1 μM range. The 5-$HT_{2A/2C}$ selective agonist DOB, as well as the serotonergic hallucinogens 5-MeO-DMT, LSD, mescaline, and psilocin stimulated the release of [^{3}H]-2-AG in a dose-dependent manner, with EC50s comparable to PI hydrolysis except in the case of psilocin (Table 4.3). The calcium ionophore, A23187 at concentrations of 3 and 6 μM, stimulated the release of [^{3}H]-2-AG to an extent comparable to 5-HT (Table 4.4). When A23187 was applied simultaneously with 10 μM 5-HT, [^{3}H]-2-AG release was greater than with 5-HT alone (Fig. 4.3).

<u>The effect of DAG lipase inhibitors, RHC80267, MAFP, and tetrahydrolipstatin on 5-HT_{2A} receptor-dependent 2-AG release</u>

The last step in the biosynthesis of 2-AG is hydrolysis of the sn1 fatty acid of DAG. In the brain, this reaction is catalyzed by sn1-specific DGLs that

Table 4.3 The Effect of 5-HT_{2A} receptor agonists on lipid metabolism in NIH3T3-5HT2A cells. EC50s for the stimulation of each lipid metabolite were determined as described under *Materials and Methods*. EC50 values represent the mean expressed in nM of three to six separate experiments. SEM values were excluded for clarity, although all were below 25% of the mean. Intrinsic activities (IA) are relative to basal and 10 μM 5-HT controls and expressed as % 5-HT.

	2-AG Release		IP Prod		AA Release[a]		PtBu Prod.	
Drug	**EC50**	**IA**	**EC50**	**IA**	**EC50**	**IA**	**EC50**	**IA**
5-HT	388	100	120[b]	100[b]	83[b]	100[b]	149	100
5-MeODMT	2820	74	2400[b]	99[b]	190[b]	70[b]	nd	nd
DOB	160	41	72[b]	79[b]	15[b]	75[b]	50	48
LSD	22	28	10[b]	22[b]	20[b]	56[b]	9.7	40
Mescaline	10400	34	11300	86	nd	nd	nd	nd
Psilocin	720	33	2300[b]	46[b]	86[b]	42[b]	nd	nd

[a] Eicosanoid Release

[b] Data taken from Kurrasch-Orbaugh (2002).

nd Not Determined

Table 4.4 The effect of inhibitors and activators on basal and 5-HT-dependent 2-AG release. The data were normalized to a basal (0%) and 10 μM 5-HT (100%) control for each experiment. Values represent the normalized mean ± (SEM) of three to six separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data.

Drug	Concentration (μM)	Drug Alone	Drug + 5-HT (10 μM)
M100907	1	nd	1.2 (2.8)*
	10	-9 (5)#	-8 (5)*
A23187	3	90 (12)#	145 (5)*
	6	102 (11)#	198 (10)*
RHC80267	10	nd	92 (3)
	100	-10 (12)	5 (11)*
THL	0.1	nd	70 (9)*
	1	-9 (5)#	4 (15)*
MAFP	5	1 (2)	25 (4)*
	10	nd	16 (4)*
AM404	10	13 (4)#	272 (9)*
	100	15 (4)#	595 (19)*
U597	0.1	nd	369 (19)*
	1	23 (4)#	405 (33)*
U602	10	nd	152 (8)*
	100	-6 (1)	159 (15)*

# $P < 0.05$ compared with a basal control

* $P < 0.05$ compared with a 5-HT control

nd Not Determined

Table continued on the following page

Table 4.4 (continued). The effect of inhibitors and activators on basal and 5-HT-dependent 2-AG release. The data were normalized to a basal (0%) and 10 μM 5-HT (100%) control for each experiment. Values represent the normalized mean ± (SEM) of three to six separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data.

Drug	Concentration (μM)	Drug Alone	Drug + 5-HT (10 μM)
U73122	10	1 (4)	4 (1)*
Et-18-OCH_3	1	nd	91 (10)
	50	-17 (1)#	56 (1)*
D609	1	nd	99 (10)
	10	-5 (3)	194 (11)*
m-3M3FBS	50	189 (35)#	174 (18)*
Brefeldin A	100	21 (5)#	150 (25)
Propranolol	100	36 (3)#	103 (29)
n-Butanol	0.5% v/v	9 (1)#	233 (36)*
Staurosporine	0.1	-1 (2)	252 (54)*
PMA	0.1	11 (11)	86 (16)

# $P < 0.05$ compared with a basal control

* $P < 0.05$ compared with a 5-HT control

nd Not Determined

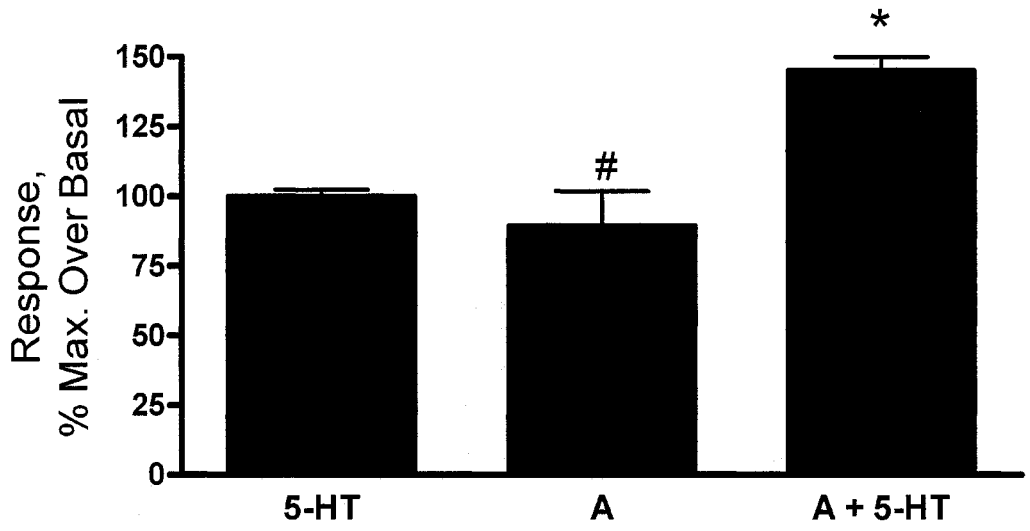


Figure 4.3 The effect of A23187 (A) on basal and 5-HT-stimulated [^{3}H]-2-AG release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 10 μM 5-HT alone (over basal; leftmost bar) was set to 100% and the treatment data are normalized to that response. The middle bar shows the effect of 3 μM of the calcium ionophore A23187 on [^{3}H]-2-AG release. The right bar shows the effect when both 3 μM A23187 and 10 μM 5-HT are added together. Basal [^{3}H]-2-AG release averaged 85 ± 5 dpm and 5-HT-stimulated [^{3}H]-2-AG release averaged 236 ± 36 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. # $p < 0.05$ compared with basal control; no significant difference from 5-HT alone.

recently have been cloned and characterized (Bisogno *et al.* 2003). Inhibition of DGL should theoretically inhibit the production and release of 2-AG. As expected, in cells pre-labeled with [^{3}H]-AA, the DGL inhibitor RHC 80267, at a concentration of 100 μM, completely inhibited the 5-HT_{2A} receptor-dependent release of [^{3}H]-2-AG (Fig. 4.4). RHC 80267 failed to inhibit this response significantly at a concentration of 10 μM (Table 4.4). Additionally, THL at 1 μM and MAFP at 5 μM inhibited 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release by 96% and 75%, respectively (Fig. 4.4). A one order of magnitude lower concentration of THL only inhibited the release of [^{3}H]-2-AG by 30% and 10 μM MAFP failed to inhibit [^{3}H]-2-AG release significantly more than 5 μM MAFP (Table 4.4). Surprisingly, only THL at 1 μM had any significant effect on basal [^{3}H]-2-AG release (Table 4.4).

<u>The effect of the endocannabinoid transport inhibitor, AM404, the monoacylglycerol lipase (MGL) inhibitor, URB602, and the fatty acid amide hydrolase (FAAH) inhibitor, URB597, on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release</u>

If 2-AG is produced following 5-HT_{2A} receptor activation, compounds that affect endocannabinoid uptake and hydrolysis would be expected to yield higher measured [^{3}H]-2-AG levels following receptor activation. After demonstrating that

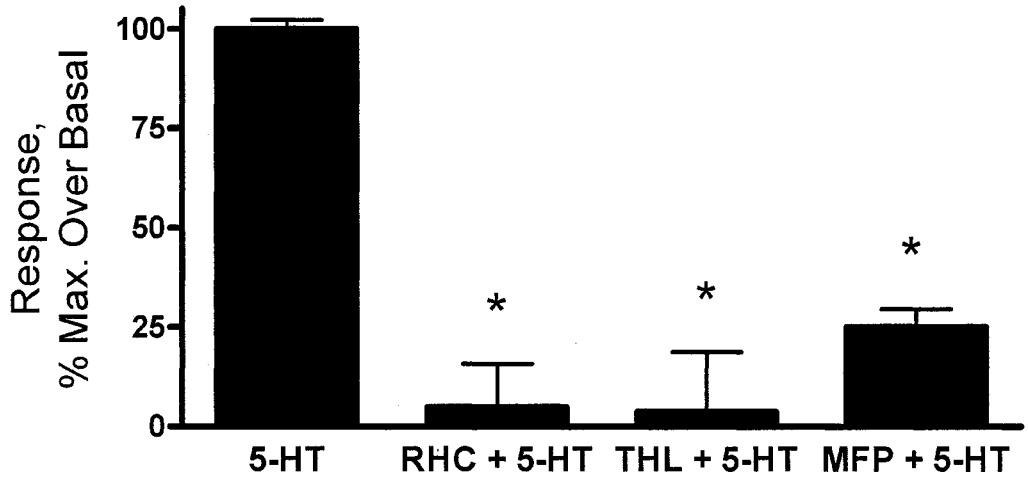


Figure 4.4 The effect of DGL inhibition on 5-HT-stimulated [^{3}H]-2-AG release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-2-AG release. The 2nd bar from the left shows the effect of 100 μM RHC80267 (RHC) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. The 3rd bar from the left shows the effect of 1 μM THL on 10 μM 5-HT-stimulated [^{3}H]-2-AG release, and the rightmost bar shows the effect of 5 μM MAFP (MFP) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. Basal [^{3}H]-2-AG release averaged 93 ± 10 dpm and 5-HT-stimulated [^{3}H]-2-AG release averaged 207 ± 15 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control.

DGL activation was required for 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release, the effect of endocannabinoid transport, FAAH, and MGL inhibition on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release was explored. Pretreatment with the endocannabinoid transport inhibitor AM404 at 10 μM led to a robust three-fold increase in 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release relative to 5-HT alone (Fig. 4.5), whereas AM404 by itself had only a slight effect on basal [^{3}H]-2-AG release (Table 4.4). At a higher concentration of 100 μM, AM404 increased the 5-HT response by six-fold (Table 4.2) possibly a result of the simultaneous activation of the calcium channel, vanilloid receptor 1 (VR1). Similar results were obtained when the FAAH inhibitor URB597 at 100 nM was employed. The MGL inhibitor URB602 produced a modest (ca. 50%), though significant, increase in 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release at 10 μM (Fig. 4.5). URB597 at 1 μM had a significant effect on basal [^{3}H]-2-AG release (Table 4.4) and was the only inhibitor to significantly inhibit 5-HT_{2A} receptor-dependent [^{3}H]-AA release (53 ± 8%). URB602 at 100 μM had no effect (116 ± 14%), and AM404 at 100 μM significantly potentiated (569 ± 47%) 5-HT_{2A} receptor-dependent [^{3}H]-AA release, possibly a result of VR1-dependent calcium influx.

The effect of PLC inhibition on 5-HT_{2A} receptor-dependent release of [^{3}H]-2-AG

One of the products of PLC activation is DAG. Hypothesizing that the

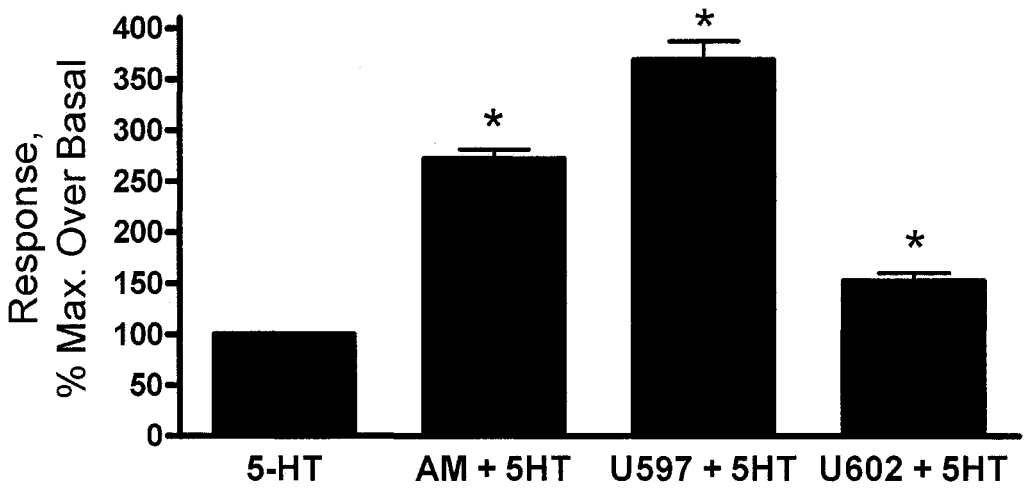


Figure 4.5 The effect of endocannabinoid transport, FAAH, and MGL inhibition on 5-HT stimulated [^{3}H]-2-AG release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-2-AG release. The 2nd bar from the left shows the effect of 10 μM AM404 (AM) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. The 3rd bar from the left shows the effect of 100 nM URB597 (U597) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. The rightmost bar shows the effect of 10 μM URB602 (U602) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. Basal [^{3}H]-2-AG release averaged 85 ± 9 dpm and 5-HT-stimulated [^{3}H]-2-AG release averaged 218 ± 19 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control.

DAG required as a substrate by DGL was derived from 5-HT_{2A} receptor dependent PLC activation, the effect of the PLC inhibitor U73122 on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release was assessed. The release of [^{3}H]-2-AG was completely inhibited by 10 μM U73122 (Fig. 4.6), previously shown to inhibit 5-HT_{2A} receptor-dependent IP accumulation completely in this cell line (Kurrasch-Orbaugh 2002). In order to confirm our results with U73122, the effect of the PI-PLC-selective inhibitor Et-18-OCH_3 on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release also was determined. Et-18-OCH_3 at a concentration of 50 μM, previously shown to completely inhibit 5-HT_{2A} receptor-dependent IP accumulation in this cell line (Kurrasch-Orbaugh 2002), inhibited the release of [^{3}H]-2-AG by almost 50% (Fig. 4.6). Curiously unlike U73122, Et-18-OCH_3 did not fully inhibit [^{3}H]-2-AG release. Because it has been reported that Et-18-OCH_3 increases cytosolic calcium concentrations in Madin Darby canine kidney cells (Jan *et al.* 1999), the possibility existed that Et-18-OCH_3 was partially stimulating 2-AG release through a calcium-dependent mechanism, while at the same time inhibiting 2-AG release by the PLC mechanism. When examined alone, however, Et-18-OCH_3 failed to stimulate [^{3}H]-2-AG release, although it significantly suppressed basal [^{3}H]-2-AG release to a modest degree (Table 4.4).

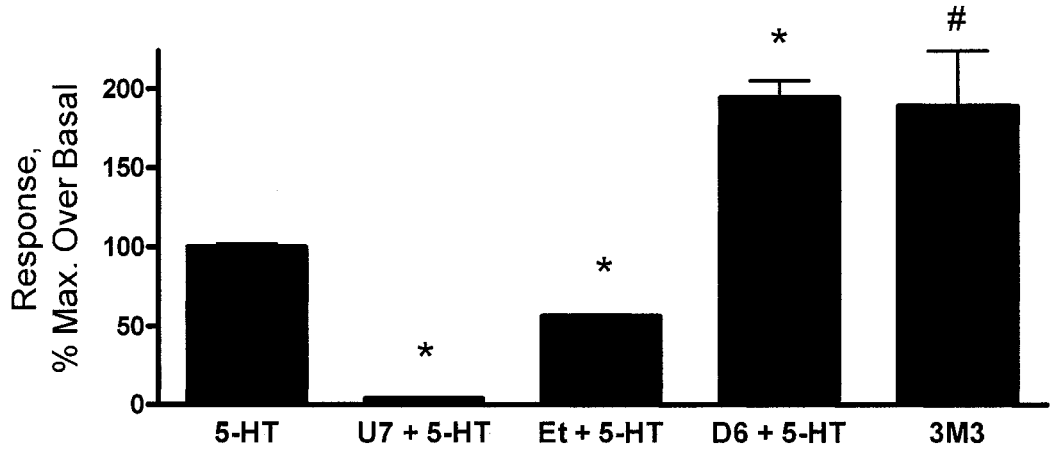


Figure 4.6 The effects of PLC inhibition on 5-HT-stimulated [^{3}H]-2-AG release and the purported PLC activator m-3M3FBS on [^{3}H]-2-AG release in NIH3T3-5-HT_{2A} cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-2-AG release. The 2nd bar from the left shows the effect of 10 μM U73122 (U7) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. The 3rd bar from the left shows the effect of 50 μM Et-18-OCH_3 (Et) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. The 4th bar from the left shows the effect of 10 μM D609 (D6) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. The rightmost bar shows the effect of 50 μM m-3M3FBS (3M3) on [^{3}H]-2-AG release. Basal [^{3}H]-2-AG release averaged 82 ± 5 dpm and 5-HT-stimulated [^{3}H]-2-AG release averaged 250 ± 25 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. # $p < 0.05$ compared with basal control.

The effect of PC-PLC-selective inhibition on 5-HT_{2A} receptor-dependent release of [^{3}H]-2-AG

Having observed only a 50% inhibition of 5-HT_{2A} receptor-dependent 2-AG release, the possibility remained that another enzymatic pathway was providing DAG for 2-AG biosynthesis. The PC-PLC pathway was a likely candidate, considering that activation of the MAPK pathway has been demonstrated following 5-HT_{2A} receptor activation in this cell line (Kurrasch-Orbaugh *et al.* 2003a). To explore the possibility that 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release was dependent on DAG produced following the activation of PC-PLC, the PC-PLC-specific inhibitor, D609 was employed. Surprisingly, at a concentration of 10 μM D609 potentiated the 5-HT_{2A} receptor-dependent release of [^{3}H]-2-AG (Fig. 4.6). A similar potentiation was observed when 100 μM D609 was used (data not shown). Examined at a concentration of 1 μM, however, the potentiating effect of D609 was not observed (Table 4.4). Because D609 is reported to be a competitive inhibitor of PC-PLC with a K_i of 6.4 μM (Amtmann 1996), the 10 μM and 100 μM concentrations of D609 are likely the most relevant. Based on these results, it is possible that the PC-PLC pathway exerts a negative influence on 2-AG levels.

The effect of the purported PLC activator, m-3M3FBS, on the release of [^{3}H]-2-AG

The purported PLC activator m-3M3FBS at a concentration of 50 μM (Bae *et al.* 2003) stimulated a greater release of [^{3}H]-2-AG than did 5-HT (Fig. 4.6). As there is some controversy concerning the mechanism of m-3M3FBS (Krjukova *et al.* 2004), the ability of a range of concentrations (1 to 100 μM) of m-3M3FBS to stimulate the production of inositol phosphates after 10 min of stimulation was measured. Surprisingly, but in agreement with the results of Krjukova *et al.* (2004), m-3M3FBS did not stimulate inositol phosphate accumulation within the 10 min time frame when 2-AG was released (Fig. 4.7), suggesting that m-3M3FBS does not directly activate PI-PLC but may indirectly activate PLC at a later time point, perhaps following calcium elevation.

The effect of inhibitors of PLD-phosphatidic acid hydrolase (PAH) pathway-dependent DAG formation on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release

Having demonstrated that 5-HT-stimulated release of [^{3}H]-2-AG was completely blocked by DGL inhibitors and partially blocked by the PI-PLC inhibitor Et-18-OCH_3, the possibility remained that [^{3}H]-2-AG release was dependent on DAG produced following 5-HT_{2A} receptor-dependent PLD and subsequent PAH activation. Therefore, cells were first pretreated with 100 μM brefeldin A, which has been reported to inhibit 5-HT_{2A} receptor-dependent PLD

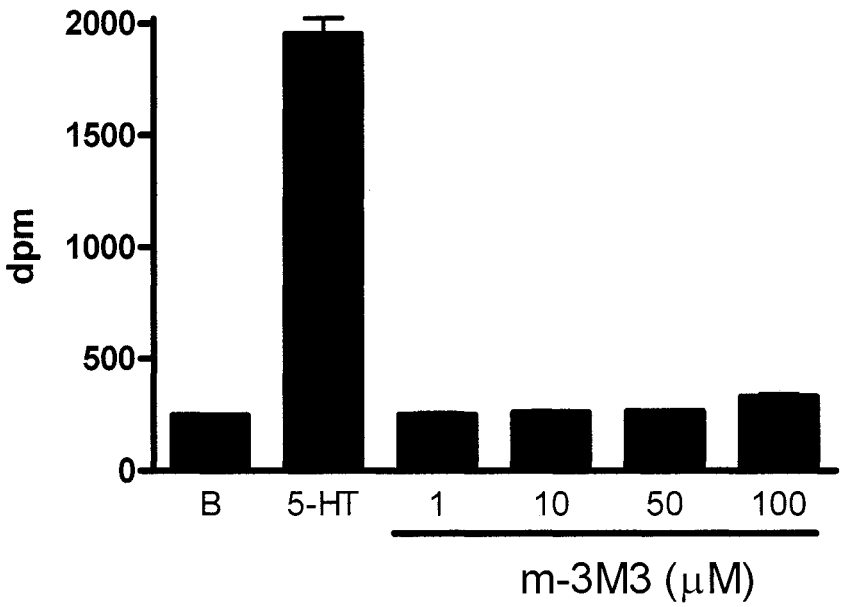


Figure 4.7 The effect of m-3M3FBS, the purported PLC activator, on inositol phosphate accumulation. Cells were incubated with [^{3}H]-myoinositol for 18 hours prior to the experiment. The leftmost bar illustrates inositol phosphate accumulation following 10 min stimulation with 10 μM 5-HT. Note that this treatment time is the same as used in all the 2-AG release experiments. The four leftmost bars show the effect of 1-100 μM m-3M3FBS (m-3M3) on inositol phosphate accumulation. Compare this result with the effect of m-3M3FBS on 2-AG release (Fig. 4.6). Basal (B) IP accumulation averaged 246 ± 6 dpm and 5-HT-stimulated IP accumulation averaged 1951 ± 49 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data.

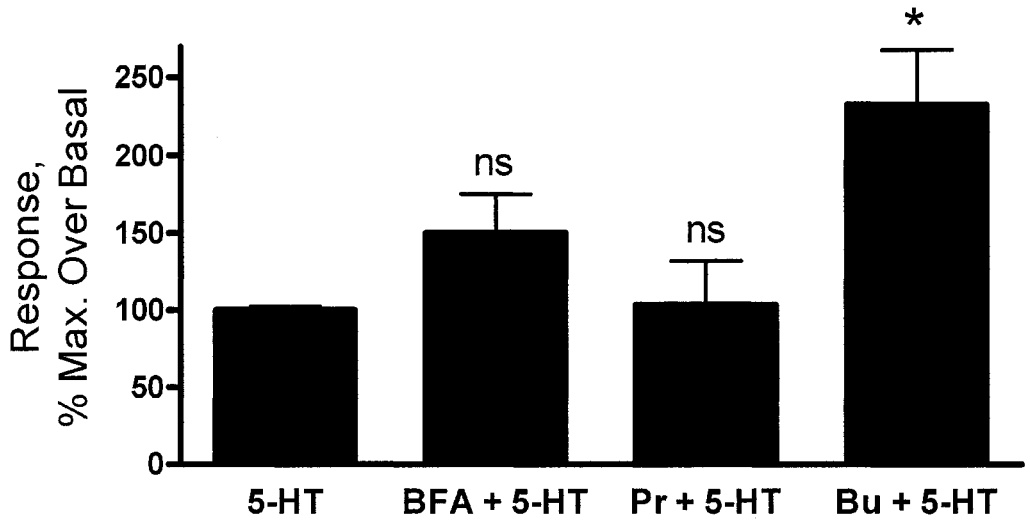


Figure 4.8 The effect of inhibitors of the PLD-PAH pathway on 5-HT stimulated [^{3}H]-2-AG release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-2-AG release. The 2nd bar from the left shows the effect of 100 μM brefeldin A (BFA) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. Compare this result with the effect of 100 μM brefeldin A on PtBu accumulation (Fig. 4.9) and [^{3}H]-AA release (Fig. 4.16). The 3rd bar from the left shows the effect of 100 μM propranolol (Pr) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. The rightmost bar shows the effect of 0.5% n-butanol (Bu) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. Basal [^{3}H]-2-AG release averaged 83 ± 4 dpm and 5-HT-stimulated [^{3}H]-2-AG release averaged 193 ± 14 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. ns $p > 0.05$ compared with 5-HT control.

activity by 50% in COS-7 cells (Robertson *et al.* 2003). Brefeldin A had no significant effect on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release at this concentration (Fig. 4.8), but reduced PLD activity by 50% (Fig. 4.9). Likewise, 100 μM propranolol, reported to inhibit 2-AG production by 50% in N18TG2 cells (Bisogno *et al.* 1999), had no effect on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release (Fig. 4.8).

In the second step of the PLD catalysis mechanism, a molecule of water completes the catalytic cycle by liberating free PA from a serine residue. Primary alcohols compete for water in this mechanism, liberating instead a PA-alcohol derivative, and effectively inhibiting the production of PA and downstream DAG. When we included 0.5% n-butanol (BuOH) in our assay we unexpectedly found that it had a significant potentiating effect on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release (Fig. 4.8). Although it is possible that the potentiating effect of BuOH may be a result of non-specific interactions or membrane fluidity issues, 0.5% BuOH had only a slight effect on basal [^{3}H]-2-AG release (Table 4.4). Nonetheless, the combined results support the hypothesis that DAG generated through the 5-HT_{2A} receptor-dependent PLD pathway does not contribute to 2-AG production. In the absence of 5-HT we observed a slight but significant increase in basal [^{3}H]-2-AG release for all three inhibitors of the PLD pathway (Table 4.4), a result consistent with the above hypothesis. It is possible that this

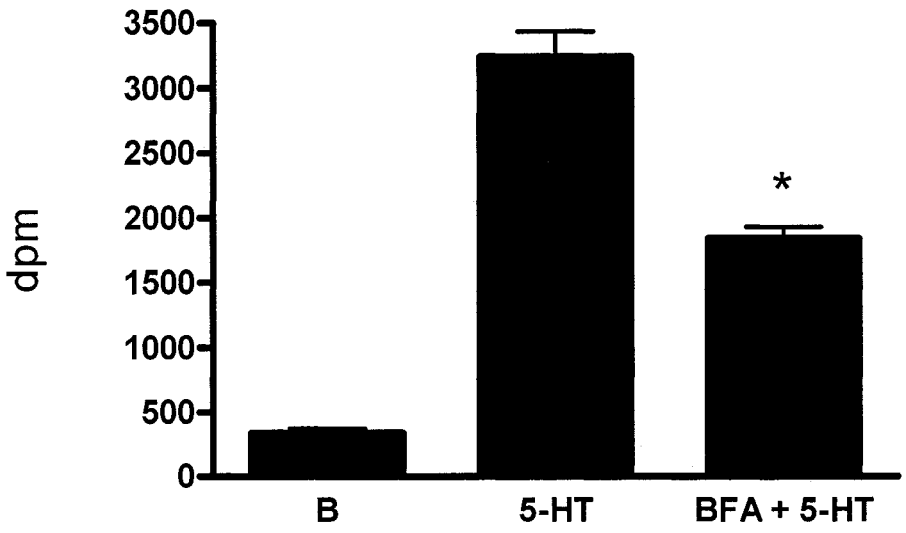


Figure 4.9 The effect of brefeldin A on 5-HT stimulated [^{3}H]-PtBu accumulation in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-palmitic acid for 24 hours prior to the experiment. The left bar shows the basal (B) level of [^{3}H]-PtBu accumulation. The middle bar shows the effect of 10 μM 5-HT on [^{3}H]-PtBu accumulation. The right bar shows the effect of 100 μM brefeldin A (BFA) on 10 μM 5-HT-stimulated [^{3}H]-PtBu accumulation. Basal [^{3}H]-PtBu accumulation averaged 340 ± 36 dpm and 5-HT-stimulated [^{3}H]-PtBu accumulation averaged 3241 ± 196 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control.

pathway, similar to the PC-PLC pathway, exerts a negative influence on 2-AG production or, alternatively, a positive influence on 2-AG hydrolysis in our cell line. Indeed the latter case may be true, as all three treatments significantly inhibited [^{3}H]-AA release (See results for specific aim 3).

The effect of protein kinase C (PKC) inhibition and stimulation on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release

Both calcium and DAG are produced downstream of 5-HT_{2A} receptor stimulation and play a role in the activation of PKC. Based on our data, DAG produced from either the PC-PLC pathway or the PLD-PAH pathway does not contribute to 2-AG production in the NIH3T3-5HT_{2A} cell line. Inhibitors of the PLD-PAH pathway generate a small but significant stimulation of 2-AG release over basal, while having zero or a potentiating effect on 5-HT-stimulated 2-AG release. Perhaps the PC-PLC pathway and the PLD-PAH pathway stimulate an enzyme that regulates 2-AG production or hydrolysis. It is possible that DAG produced following the activation of these enzymes leads to PKC stimulation, which negatively regulates 2-AG production, or alternatively, positively regulates 2-AG hydrolysis. The effect of PKC inhibition on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release was determined using 100 nM staurosporine. When NIH3T3-5HT_{2A} cells were pretreated with staurosporine, 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release was considerably potentiated (Fig. 4.10), while staurosporine alone had no significant effect (Table 4.4). In order to observe the reverse effect, the

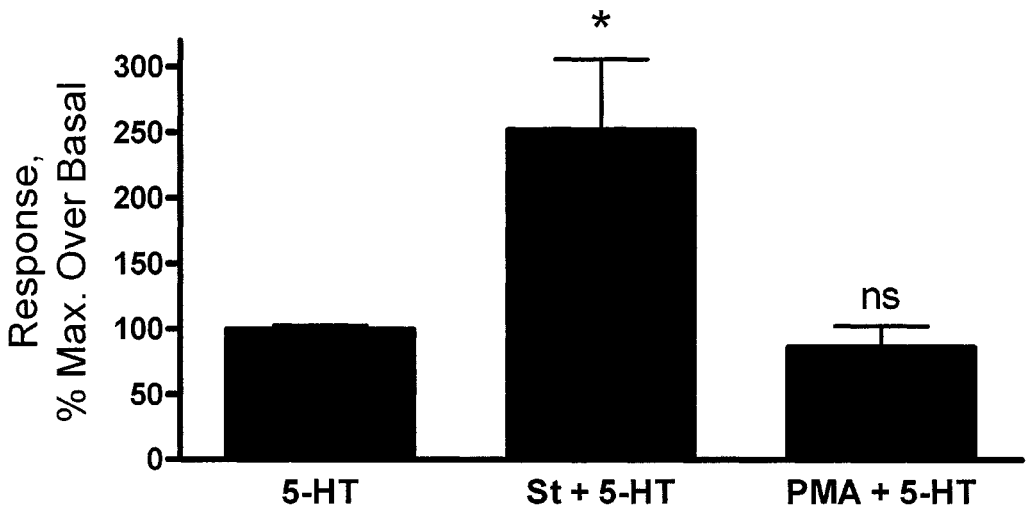


Figure 4.10 The effect of PKC inhibition and activation on 5-HT stimulated [^{3}H]-2-AG release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. The left bar illustrates the effect of 10 μM 5-HT on [^{3}H]-2-AG release. The middle bar shows the effect of 100 nM staurosporine (St) on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. Compare this result with the effect of 100 nM staurosporine on [^{3}H]-AA release (Fig. 4.17). The bar on the right shows the effect of 100 nM PMA on 10 μM 5-HT-stimulated [^{3}H]-2-AG release. Basal [^{3}H]-2-AG release averaged 98 ± 12 dpm and 5-HT-stimulated [^{3}H]-2-AG release averaged 218 ± 29 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. ns $p > 0.05$ compared with 5-HT control.

PKC activator PMA was tested on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release. Co-administration of 100 nM PMA with 10 μM 5-HT had no significant effect on 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release (Fig. 4.10), and PMA treatment alone had no significant effect on basal [^{3}H]-2-AG release (Table 4.4). As with the inhibitors of the PLD-PAH pathway, staurosporine had an inhibitory effect (50 ± 12 %) on 5-HT_{2A} receptor-dependent [^{3}H]-AA release whereas PMA had no effect (95 ± 10 %) on this response.

4.2.2. Discussion

In conclusion, from specific aim 2, the first direct evidence was presented that 5-HT_{2A} receptor stimulation leads to the release of the endocannabinoid 2-AG. Based on these experimental data, 5-HT_{2A} receptor-dependent 2-AG release is at least partially dependent on 5-HT_{2A} receptor-dependent PI-PLC activation. DAG produced from either the PC-PLC pathway or downstream of PLD production of PA and PAH-dependent hydrolysis of PA does not contribute to 2-AG biosynthesis.

For the 5-HT_{2A} receptor agonists shown in Table 4.3, the absence of large differences between EC50s for IP accumulation and 2-AG release, except in the case of psilocin, supports the hypothesis that 2-AG release is dependent on PLC activation. Total measured eicosanoid release has been shown to be PLC independent in NIH3T3-5HT_{2A} cells (Kurrasch-Orbaugh *et al.* 2003b). Although 2-AG is an eicosanoid, its production was found to be PLC dependent in this

same cell line. As total eicosanoid release is measuring a population of different lipid species that are the products of multiple enzymes competing for the same AA substrate, the differences observed between the EC50s for 2-AG release and total eicosanoid release are not surprising. Future studies should focus on identifying specific lipid species of the eicosanoid population that are released following 5-HT_{2A} receptor activation rather than total eicosanoid release.

After determining that 2-AG release was dependent on 5-HT_{2A} receptor activation, it was demonstrated that 5-HT_{2A} receptor-dependent 2-AG release was completely inhibited by the DGL inhibitor RHC80267, which was shown by Bisogno et al. (2003) to inhibit 2-AG production in enzyme assays using cloned DGLs. Additionally, MAFP, which is known to inhibit DGL (Moriyama *et al.* 1999), phospholipase A2, and FAAH, inhibited 5-HT_{2A} receptor-dependent 2-AG release by 75%. Furthermore, 1 μM THL, which was shown by Bisogno et al. (2003) to inhibit ionomycin-stimulated 2-AG production in many cell types, inhibited 5-HT_{2A} receptor-dependent 2-AG release by 96%. The endocannabinoid transport inhibitor AM404 and the FAAH inhibitor URB597 both robustly potentiated 5-HT_{2A} receptor-dependent 2-AG release, whereas the MGL inhibitor URB602 modestly increased 5-HT_{2A} receptor-dependent 2-AG release. Based on these data, [^{3}H]-2-AG produced following DGL activation in the NIH3T3-5HT_{2A} cell line is metabolized by FAAH and MGL to AA and glycerol. MGL, the main 2-AG hydrolyzing enzyme in the brain (Dinh *et al.* 2004;Gulyas *et al.* 2004;Saario *et al.* 2004) appears to provide less hydrolytic influence, although it remains questionable whether or not MGL was fully inhibited by 100 μM U602.

Perhaps when more potent MGL inhibitors become available this question can be determined.

It was next determined that 5-HT_{2A} receptor-dependent 2-AG release was partially dependent on DAG produced by PI-PLC activation. The PLC inhibitor U73122 completely inhibited 5-HT_{2A} receptor-dependent 2-AG release at a concentration sufficient to fully inhibit inositol phosphate accumulation in NIH3T3-$5HT_{2A}$ cells (Kurrasch-Orbaugh 2002). By contrast, the PI-PLC-selective inhibitor Et-18-OCH_3, at a concentration sufficient to inhibit inositol phosphate accumulation completely in NIH3T3-$5HT_{2A}$ cells (Kurrasch-Orbaugh *et al.* 2003b), reduced 5-HT_{2A} receptor-dependent 2-AG release by only 50%. U73122 alone had no effect on basal 2-AG release, whereas ET-18-OCH_3 significantly reduced basal release to a modest degree (Table 4.4).

When this study was first initiated, m-3M3FBS was described as a novel nonspecific but direct PLC activator (Bae *et al.* 2003). Recently, however, this classification has been called into question (Krjukova *et al.* 2004). The ability of m-3M3FBS to produce inositol phosphates in NIH3T3-$5HT_{2A}$ cells was next addressed. Within the 10 min time frame when m-3M3FBS strongly stimulated 2-AG release, no increase in the accumulation of inositol phosphates was observed, confirming the results of Krjukova *et al.*, (2004). Although the direct PLC stimulatory activity of m-3M3FBS is now questionable, the stimulation of 2-AG production may result from its effects on calcium homeostasis (Krjukova *et al.* 2004). Nonetheless, based on the above experiments, 5-HT_{2A} receptor

stimulation leads to the biosynthesis and release of 2-AG, at least partially through a $G_{q/11}$-PI-PLC-DGL pathway.

In some cell lines, it has been shown that 2-AG can be produced in a PLD-dependent manner (Bisogno *et al.* 1999;Carrier *et al.* 2004). The 5-HT_{2A} receptor activates PLD via the monomeric G-protein, Arf1 (Robertson *et al.* 2003). PLD catalyzes the production of phosphatidylbutanol (PtBu) when stimulated in the presence of exogenous n-butanol. After determining that brefeldin A at 100 μM reduces PtBu production by 50% following 5-HT_{2A} receptor stimulation in NIH3T3-5HT_{2A} cells (Fig. 4.10), the effect of brefeldin A on 5-HT_{2A} receptor-dependent 2-AG release was investigated. When NIH3T3-5HT_{2A} cells were treated with brefeldin A, no significant reduction in 5-HT_{2A} receptor-dependent 2-AG release was observed. Additionally, the beta adrenergic receptor antagonist propranolol, which also inhibits PAH (Perry *et al.* 1992), had no effect on 5-HT_{2A} receptor-dependent 2-AG release. When n-butanol, an inhibitor of PLD-dependent PA production was included, 5-HT_{2A} receptor-dependent 2-AG release was actually potentiated. Based on the fact that all three of these treatments failed to inhibit 5-HT_{2A} receptor-dependent 2-AG release, DAG produced from PLD and subsequent PAH activation most likely does not lead to 2-AG biosynthesis in NIH3T3-5HT_{2A} cells. Curiously, all three treatments led to a significant and selective inhibition of AA release (Fig. 4.16), while at the same time potentiating basal 2-AG release (Table 4.4).

Thus, at least 50% of 5-HT_{2A} receptor-dependent 2-AG release was dependent on PI-PLC activation but not on PLD and PAH activation. In attempts

to discover the source of the remaining 50% of 2-AG, D609, a PC-PLC-selective inhibitor with a reported K_i of 6.4 μM (Amtmann 1996) was tested in the assay. Pretreatment with 1 μM D609 had no effect on 5-HT_{2A} receptor-dependent 2-AG release, whereas 10 μM D609 actually led to a potentiation of 5-HT_{2A} receptor-dependent 2-AG release. One explanation for this observation is that DAG produced following PC-PLC activation, as with PLD/PAH activation, may lead to PKC activation, which may act either as a negative regulator of 2-AG release or as a positive regulator of 2-AG uptake and hydrolysis. This explanation assumes that the DAG produced following PC-PLC activation is spatially separated from the DAG that serves as the substrate for 2-AG biosynthesis. Indeed, the PKC inhibitor staurosporine had a similar potentiating effect on 5-HT_{2A} receptor-dependent 2-AG release, while at the same time inhibiting AA release by 50%, as did the three PLD-PAH pathway inhibitors. This speculation is confounded, however, by the fact that PMA had no effect on 5-HT_{2A} receptor-dependent 2-AG or AA release. It is possible that direct activation of DGL by the 5-HT_{2A} receptor through an unknown mechanism yields the other 50% of released 2-AG, as a small pool of DAG exists in the membrane of unstimulated NIH3T3 cells (Florin-Christensen *et al.* 1992). In that case, direct DGL activation would shift the equilibrium of upstream enzymes by reducing the pool of DAG.

The neuromodulatory neurotransmitters, including serotonin (5-HT), dopamine, acetylcholine, histamine, and norepinephrine, affect cognitive functioning by activating receptors that influence excitatory circuits within the CNS (Gu *et al.* 2005). Similar to neuromodulatory neurotransmitters,

endocannabinoids appear to be tonic regulators of normal cognitive functioning (Reibaud *et al.* 1999;Di Marzo *et al.* 2000), and it is now widely believed that endocannabinoids play a major role in modulating cognitive effects, particularly working memory (Varvel and Lichtman 2002). Cannabinoids are produced postsynaptically, whereupon they migrate to presynaptic axon terminals, bind to cannabinoid sensitive sites, and lead to decreased excitatory (Kreitzer and Regehr 2001a), inhibitory (Ohno-Shosaku *et al.* 2001;Wilson and Nicoll 2001;Kreitzer and Regehr 2001b), and neuromodulatory neurotransmitter release (Gifford and Ashby, Jr. 1996;Schlicker *et al.* 1996;Cadogan *et al.* 1997;Schlicker *et al.* 1997;Nakazi *et al.* 2000;Steffens *et al.* 2003;Steffens *et al.* 2004).

At excitatory synapses, endocannabinoids inhibit glutamate release following postsynaptic depolarization/Ca^{2+}-dependent biosynthesis, retrograde transport, and activation of presynaptic CB1 receptors (Freund *et al.* 2003). Although the depolarization-dependent mechanism for endocannabinoid release was first documented, there is now evidence for an additional GPCR-$G_{q/11}$-PLC mechanism (Maejima *et al.* 2001a;Kim *et al.* 2002;Ohno-Shosaku *et al.* 2003;Jung *et al.* 2005;Maejima *et al.* 2005). A more recent study (Maejima *et al.* 2005) suggests that simultaneous depolarization and $G_{q/11}$-coupled GPCR activation is likely to be more physiologically relevant than either stimulus alone. In that study, 2-AG release was achieved by simultaneous activation of mGluR1 and depolarization with 20 mM potassium ion. Either treatment alone failed to elicit 2-AG release. PLCβ has been shown to be the focal point where $G_{q/11}$ activation and depolarization-induced calcium influx converge to enhance

endocannabinoid release (Hashimotodani *et al.* 2005). In NIH3T-5HT$_{2A}$ cells, an enhancement in 5-HT$_{2A}$ receptor-dependent 2-AG release was observed when a calcium ionophore was included. A23187 alone stimulated 2-AG release to a degree equivalent to 5-HT$_{2A}$ receptor-dependent stimulation of 2-AG release, and when applied simultaneously with 5-HT a response was observed that was greater than 5-HT alone. These results support the possibility that one of the roles of neuromodulatory neurotransmitters is to modulate depolarization/calcium-dependent endocannabinoid release.

There is now abundant evidence to support the idea that endocannabinoids and neuromodulatory neurotransmitters mutually regulate the release of each other. For example, activation of dopamine (Giuffrida *et al.* 1999), acetylcholine (Kim *et al.* 2002), and metabotropic glutamate receptors (Maejima *et al.* 2001a) has been shown to enhance endocannabinoid release. Likewise, there is evidence that endocannabinoids or CB1 agonists regulate the release of acetylcholine (Gifford and Ashby, Jr. 1996;Steffens *et al.* 2003), dopamine (Schlicker *et al.* 1996;Cadogan *et al.* 1997;Steffens *et al.* 2004), norepinephrine (Schlicker *et al.* 1997), and 5-HT (Nakazi *et al.* 2000). It is plausible that neuromodulatory neurotransmitters, through the action of postsynaptic metabotropic receptors, partially regulate synaptic activity through the differential activation or inhibition of basal and/or depolarization-induced endocannabinoid release.

CB1 receptor stimulation by endocannabinoids released following 5-HT$_{2A}$ receptor activation may exemplify a negative regulatory mechanism by which

postsynaptic GPCRs can modulate presynaptic neurotransmitter release and neuronal plasticity. With the addition of timed postsynaptic GPCR activation, serotonin and/or other neuromodulatory neurotransmitters may alter the depolarization/calcium-induced 2-AG level either by inhibiting or additively/synergistically enhancing 2-AG biosynthesis. With evidence that activation of some neuromodulatory neurotransmitter receptors enhances endocannabinoid release in the brain (Giuffrida *et al.* 1999;Kim *et al.* 2002), evidence from animal models (Darmani and Pandya 2000;Darmani 2001;Gorzalka *et al.* 2005), and the results presented here that $G_{q/11}$-coupled 5-HT_{2A} receptors stimulate 2-AG release in NIH3T3-5HT_{2A} cells, it seems plausible that 5-HT_{2A} receptor-dependent 2-AG release is physiologically relevant. In conclusion, this study demonstrates for the first time that 5-HT_{2A} receptor stimulation leads to the release of 2-AG through a mechanism that involves the activation of PI-PLC.

4.3. Serotonin 5-HT_{2A} receptor activation induces AA release through a FAAH and PLD-dependent mechanism.

Many studies have demonstrated that activation of the 5-HT_{2A} receptor stimulates the release of arachidonic acid, supposedly through the activation of PLA_2 (Felder *et al.* 1990;Berg *et al.* 1998a;Berg *et al.* 1998b;Kurrasch-Orbaugh 2002;Kurrasch-Orbaugh *et al.* 2003a;Kurrasch-Orbaugh *et al.* 2003b). Only one of these studies simultaneously measured the production of lysophospholipids along with total eicosanoid release (Felder *et al.* 1990). Measuring

lysophospholipid production is currently challenging and not suited to high-throughput assays. Many laboratories, therefore, simply measure total [^{3}H]-eicosanoid release from cells pretreated with [^{3}H]-AA and conclude that the measured radioactivity is PLA_2-dependent AA release. Unfortunately, this practice leads to erroneous conclusions because PLA_2 is not the only enzyme that can lead to AA release. There is always the unchecked possibility that many eicosanoids are released simultaneously. Testing for lysophospholipid production and correlating it with eicosanoid release, although difficult, is currently the only way to control for these uncertainties.

As demonstrated under specific aim 2, NIH3T3-5HT_{2A} cells release 2-AG and free AA when stimulated by 5-HT_{2A} receptor agonists. From NIH3T3-5HT_{2A} cells stimulated for 10 minutes, $CHCl_3$:MeOH extracted [^{3}H]-lipids accounted for approximately 90% of the total radio-labeled eicosanoids released into the cellular medium. The real percentage [^{3}H]-lipids released was likely higher as the above percentages assume 100% extraction efficiency. Approximately 18% of the $CHCl_3$:MeOH extracted [^{3}H]-lipids were located at the baseline of the TLC lane and remain unidentified. The remaining lipids were positively identified as 2-AG (28%) and AA (56%). With the knowledge that 2-AG was being produced by this cell line it was likely that at least a portion of the AA was the hydrolysis product of 2-AG and, therefore, not derived from PLA_2 activation. Alternative sources of AA biosynthesis were sought by looking at the changes in AA release after pretreating NIH3T3-5HT_{2A} cells with inhibitors of different signaling pathways.

4.3.1. Results

The effect of 5-HT_{2A} receptor activation on [^{3}H]-AA release

As mentioned in the results section of the previous specific aim, AA release was observed simultaneously with 2-AG release. The 5-HT-stimulated release of [^{3}H]-AA was dose dependent (Fig. 4.11) and was completely inhibited by the selective 5-HT_{2A} receptor antagonist M100907 (Fig. 4.11 inset) (Sorensen *et al.* 1993;Kehne *et al.* 1996). The calcium ionophore, A23187, at a concentration of 3 μM, stimulated the release of [^{3}H]-AA to an extent comparable to 5-HT (Table 4.5). When A23187 was applied simultaneously with 10 μM 5-HT, [^{3}H]-AA release was greater than with 5-HT alone (Fig. 4.12).

The effect of the DAG lipase inhibitors, RHC 80267, THL, and MAFP on 5-HT_{2A} receptor-dependent [^{3}H]-AA release

After demonstrating that the observed AA release was 5-HT_{2A} receptor dependent, DAG lipase inhibitors were used to assess the role of this enzyme on AA release. Both RHC 80267 and MAFP fully inhibited 5-HT_{2A} receptor-dependent [^{3}H]-AA release at concentrations of 100 μM and 10 μM, respectively (Fig. 4.13). Although a lower 10 μM concentration of RHC 80267 failed to inhibit 5-HT_{2A} receptor-dependent [^{3}H]-AA release, 5 μM MAFP still inhibited the response by 63%. Curiously, THL at 1 μM and 100 nM had no significant effect

Table 4.5 The effect of inhibitors and activators on basal and 5-HT-dependent AA release. The data were normalized to a basal (0%) and 10 μM 5-HT (100%) control for each experiment. Values represent the normalized mean ± (SEM) of three to six separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data.

Drug	Concentration (μM)	Drug Alone	Drug + 5-HT (10 μM)
M100907	10	-17 (3)#	-12 (3)*
A23187	3	94 (27)#	160 (24)*
RHC80267	100	-66 (44)#	-9 (10)*
THL	0.1	nd	91 (4)
	1	-4 (3)	104 (16)
MAFP	5	-13 (4)#	37 (4)*
	10	nd	1 (6)*
AM404	10	nd	217 (7)*
	100	28 (6)#	569 (47)*
U597	1	10 (12)	53 (8)*
U602	100	7 (6)	116 (14)

# P < 0.05 compared with a basal control

* P <0.05 compared with a 5-HT control

nd Not Determined

Table continued on the following page

Table 4.5 (continued). The effect of inhibitors and activators on basal and 5-HT-dependent AA release. The data were normalized to a basal (0%) and 10 μM 5-HT (100%) control for each experiment. Values represent the normalized mean ± (SEM) of three to six separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data.

Drug	Concentration (μM)	Drug Alone	Drug + 5-HT (10 μM)
U73122	10	-13 (4)#	-14 (4)*
Et-18-OCH_3	50	-17 (7)#	133 (24)
D609	10	0.1 (2)	121 (30)
	50	13 (2)#	73 (14)
Brefeldin A	100	-7 (7)	45 (12)*
Propranolol	100	-3 (6)	32 (20)*
n-Butanol	0.5% v/v	1 (2)	53 (5)*
Staurosporine	0.1	3 (3)	50 (12)*
PMA	0.1	9 (9)	94 (10)

# $P < 0.05$ compared with a basal control

* $P < 0.05$ compared with a 5-HT control

nd Not Determined

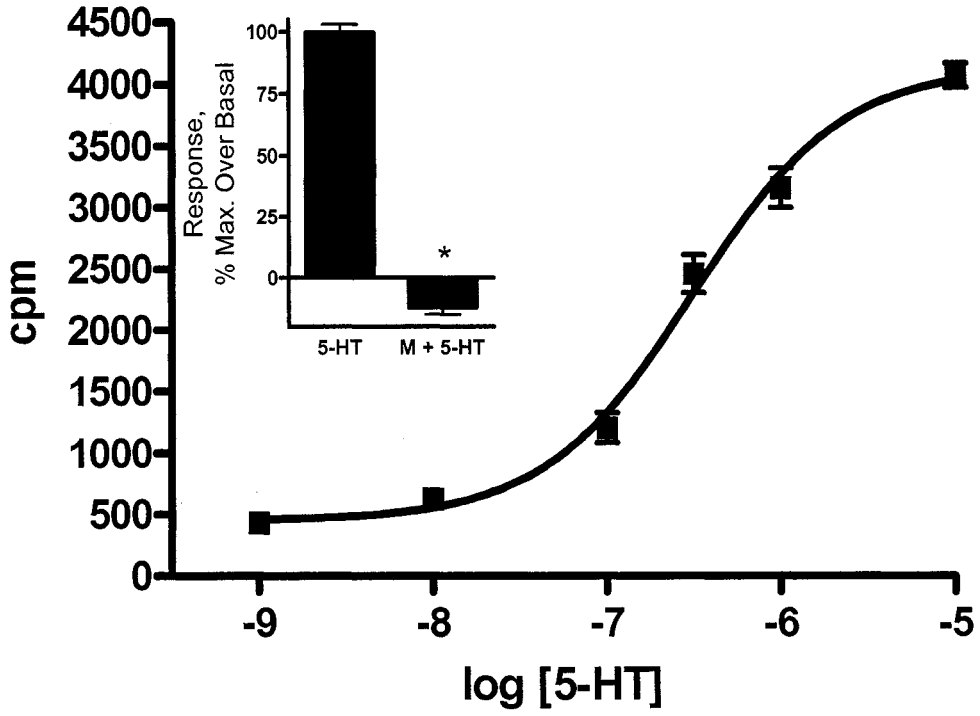


Figure 4.11 The release of AA stimulated by the 5-HT_{2A} receptor is dose-dependent. NIH3T3-5-HT_{2A} cells were incubated with [^{3}H]-AA for 24 hours prior to experiments. The curve illustrates one typical experiment showing [^{3}H]-AA release following 10 min stimulation with serial dilutions of 5-HT. The inset graph illustrates the effect of 1 μM M100907 on 10 μM 5-HT-stimulated [^{3}H]-AA release. Basal [^{3}H]-AA release averaged 621 ± 75 dpm and 5-HT-stimulated [^{3}H]-AA release averaged 2352 ± 432 dpm. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control.

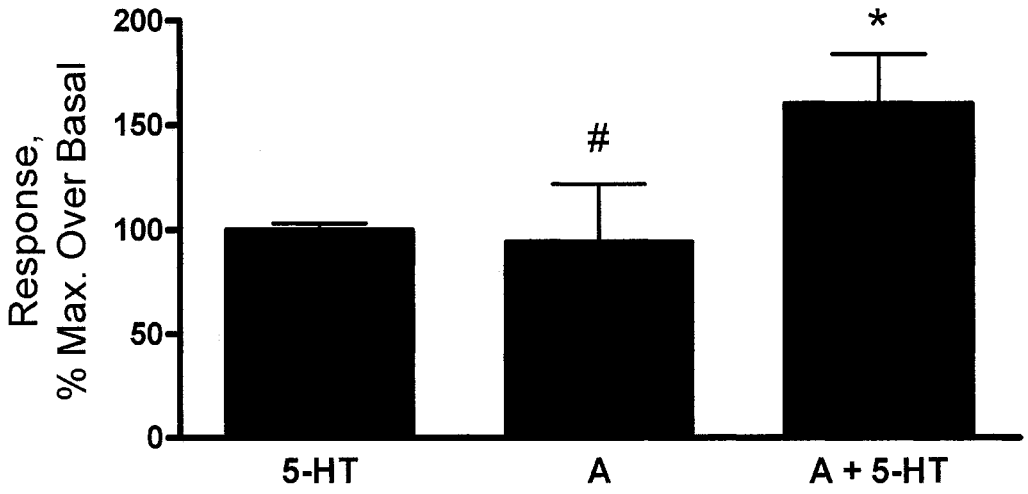


Figure 4.12 The effect of A23187 (A) on basal and 5-HT-stimulated [^{3}H]-AA release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 10 μM 5-HT alone (over basal; leftmost bar) was set to 100% and the treatment data are normalized to that response. The middle bar shows the effect of 3 μM of the calcium ionophore A23187 on [^{3}H]-AA release. The right bar shows the effect when both 3 μM A23187 and 10 μM 5-HT are added together. Basal [^{3}H]-AA release averaged 660 ± 74 dpm and 5-HT-stimulated [^{3}H]-AA release averaged 2730 ± 427 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. # $p < 0.05$ compared with basal control; no significant difference from 5-HT alone.

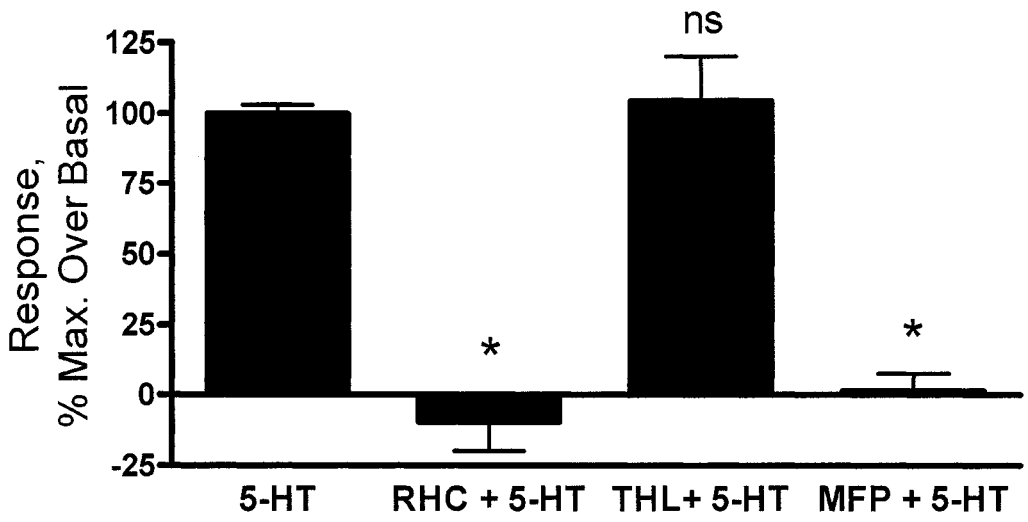


Figure 4.13 The effect of DGL inhibition on 5-HT-stimulated [^{3}H]-AA release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-AA release. The 2nd bar from the left shows the effect of 100 μM RHC80267 (RHC) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The 3rd bar from the left shows the effect of 1 μM THL on 10 μM 5-HT-stimulated [^{3}H]-AA release, and the rightmost bar shows the effect of 5 μM MAFP (MFP) on 10 μM 5-HT-stimulated [^{3}H]-AA release. Basal [^{3}H]-AA release averaged 412 ± 62 dpm and 5-HT-stimulated [^{3}H]-AA release averaged 1569 ± 225 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. ns $p > 0.05$ compared with 5-HT control.

on 5-HT_{2A} receptor-dependent [^{3}H]-AA release, whereas at the 1 μM concentration it completely inhibited 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release (Fig. 4.4). RHC 80267 strongly inhibited basal [^{3}H]-AA release whereas MAFP had a slight effect and THL was without action (Table 4.5).

The effect of the endocannabinoid transport inhibitor, AM404, the monoacylglycerol lipase (MGL) inhibitor, URB602, and the fatty acid amide hydrolase (FAAH) inhibitor, URB597, on 5-HT_{2A} receptor-dependent [^{3}H]-AA release

It has been demonstrated that in the NIH3T3-5HT_{2A} cell line 5-HT_{2A} receptor stimulation leads to 2-AG release. MGL, the main 2-AG hydrolyzing enzyme, has been reported to be in the brain (Dinh *et al.* 2004;Gulyas *et al.* 2004;Saario *et al.* 2004), although in the NIH3T3-5HT_{2A} cell line, as demonstrated under specific aim 2, FAAH has hydrolytic activity towards 2-AG. Theoretically, a selective inhibitor of 2-AG hydrolysis and/or uptake should inhibit [^{3}H]-AA release, while at the same time increasing the amount of observable [^{3}H]-2-AG. Although all three of the compounds tested significantly potentiated 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release, only the FAAH inhibitor U597 simultaneously inhibited AA release by approximately 50% (Fig. 4.14). At a concentration of 10 μM the endocannabinoid transport inhibitor, AM404, significantly potentiated 5-HT_{2A} receptor-dependent [^{3}H]-AA release whereas 100

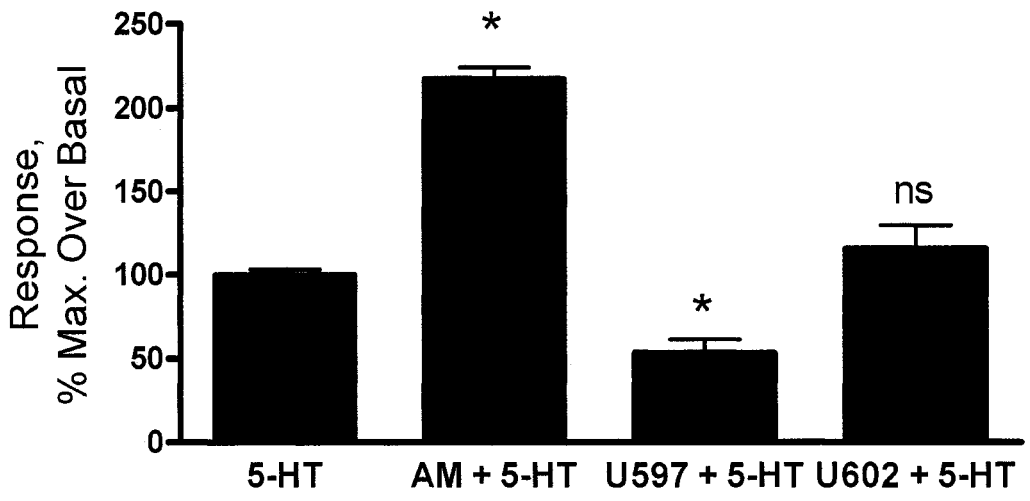


Figure 4.14 The effect of endocannabinoid transport, FAAH, and MGL inhibition on 5-HT stimulated [^{3}H]-AA release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-AA release. The 2nd bar from the left shows the effect of 10 μM AM404 (AM) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The 3rd bar from the left shows the effect of 100 nM URB597 (U597) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The rightmost bar shows the effect of 10 μM URB602 (U602) on 10 μM 5-HT-stimulated [^{3}H]-AA release. Basal [^{3}H]-AA release averaged 464 ± 118 dpm and 5-HT-stimulated [^{3}H]-AA release averaged 1781 ± 333 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. ns $p > 0.05$ compared with 5-HT control.

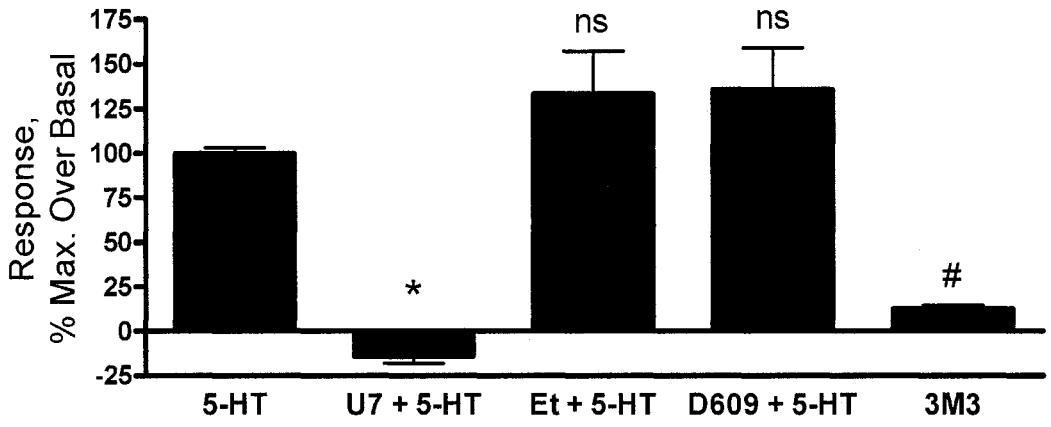


Figure 4.15 The effects of PLC inhibition on 5-HT-stimulated [^{3}H]-AA release and the purported PLC activator m-3M3FBS on [^{3}H]-AA release in NIH3T3-5-HT_{2A} cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-AA release. The 2nd bar from the left shows the effect of 10 μM U73122 (U7) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The 3rd bar from the left shows the effect of 50 μM Et-18-OCH_3 (Et) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The 4th bar from the left shows the effect of 10 μM D609 (D6) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The rightmost bar shows the effect of 50 μM m-3M3FBS (3M3) on [^{3}H]-AA release. Basal [^{3}H]-AA release averaged 630 ± 120 dpm and 5-HT-stimulated [^{3}H]-AA release averaged 1511 ± 275 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. # $p < 0.05$ compared with basal control. ns $p > 0.05$ compared with 5-HT control.

μM U602 had no significant effect (Fig. 4.14). AM404 had a slight effect on basal [^{3}H]-AA release (Table 4.5).

The effect of PLC inhibitors and the purported PLC activator on 5-HT_{2A} receptor-dependent release of [^{3}H]-AA

Results with the PLC inhibitors were mixed. Whereas 10 μM U73122 completely inhibited 5-HT_{2A} receptor-dependent [^{3}H]-AA release, both 50 μM Et-18-OCH_3 and 10 μM D609 failed to have significant effects on the response (Fig. 4.15). Interestingly, the purported PLC activator m-3M3FBS that robustly stimulated [^{3}H]-2-AG release (Fig. 4.6), while failing to simultaneously produce inositol phosphates (Fig. 4.7), had only a minor although significant effect on basal [^{3}H]-AA release (Fig. 4.15). Both U73122 and Et-18-OCH_3 significantly inhibited basal [^{3}H]-AA release, whereas D609 was without effect (Table 4.5).

The effect of inhibitors of PLD-phosphatidic acid hydrolase (PAH) pathway-dependent DAG formation on 5-HT_{2A} receptor-dependent [^{3}H]-AA release

Possibly the least ambiguous results with the AA release assays were with the inhibitors of the PLD pathway. All three methods used to inhibit the PLD pathway resulted in significant decreases in [^{3}H]-AA release. Pretreating the cells with 100 μM Brefeldin A, 100 μM propranolol, or 0.5% n-butanol significantly

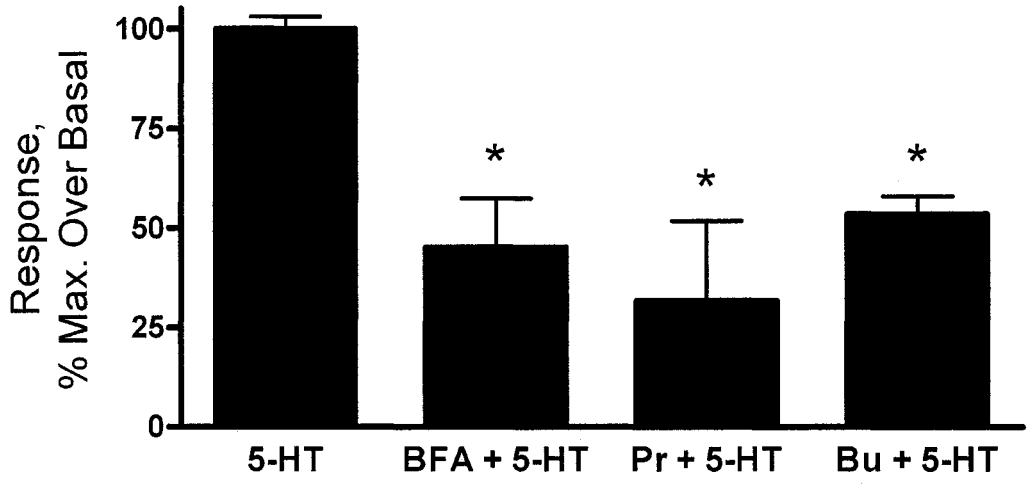


Figure 4.16 The effect of inhibitors of the PLD-PAH pathway on 5-HT stimulated [^{3}H]-AA release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. The leftmost bar illustrates the effect of 10 μM 5-HT on [^{3}H]-AA release. The 2nd bar from the left shows the effect of 100 μM brefeldin A (BFA) on 10 μM 5-HT-stimulated [^{3}H]-AA release. Compare this result with the effect of 100 μM brefeldin A on PtBu accumulation (Fig. 4.10) and 2-AG release (Fig. 4.9). The 3rd bar from the left shows the effect of 100 μM propranolol (Pr) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The rightmost bar shows the effect of 0.5% n-butanol (Bu) on 10 μM 5-HT-stimulated [^{3}H]-AA release. Basal [^{3}H]-AA release averaged 552 ± 41 dpm and 5-HT-stimulated [^{3}H]-AA release averaged 1754 ± 246 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control.

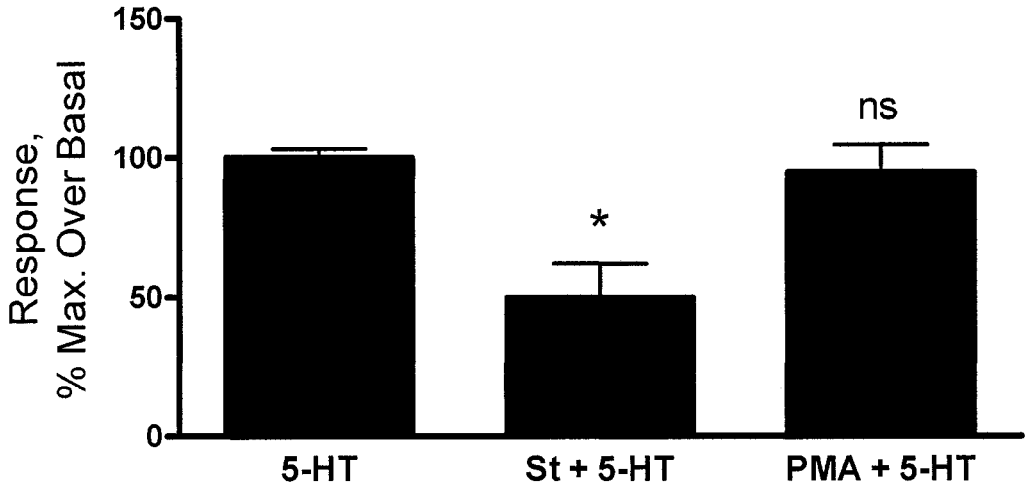


Figure 4.17 The effect of PKC inhibition and activation on 5-HT stimulated [^{3}H]-AA release in NIH3T3-5-HT$_{2A}$ cells. Cells were incubated with [^{3}H]-AA for 24 hours prior to the experiment. The effect of 5-HT alone (over basal) was set to 100% and the treatment data are normalized to that response. The left bar illustrates the effect of 10 μM 5-HT on [^{3}H]-AA release. The middle bar shows the effect of 100 nM staurosporine (St) on 10 μM 5-HT-stimulated [^{3}H]-AA release. The bar on the right shows the effect of 100 nM PMA on 10 μM 5-HT-stimulated [^{3}H]-AA release. Basal [^{3}H]-AA release averaged 353 ± 114 dpm and 5-HT-stimulated [^{3}H]-AA release averaged 1254 ± 137 dpm. The data represent the mean ± S.E.M. for at least three separate experiments. A paired t-test was used to determine significant differences between drug and control counts in raw dpm data. *$p < 0.05$ compared with 5-HT control. ns $p > 0.05$ compared with 5-HT control.

decreased 5-HT_{2A} receptor-dependent [^{3}H]-AA release by 55 %, 68 %, and 47 %, respectively (Fig. 4.16). All three inhibitors lacked significant effects on basal [^{3}H]-AA release (Table 4.5). These results are a clear indication that 5-HT_{2A} receptor-dependent [^{3}H]-AA release is at least partially dependent on PLD activation.

The effect of protein kinase C (PKC) inhibition and stimulation on 5-HT_{2A} receptor-dependent [^{3}H]-AA release

As demonstrated in the results under specific aim 2, when NIH3T3-5HT_{2A} cells were pretreated with the PKC inhibitor staurosporine, 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release was considerably potentiated (Fig. 4.10), whereas staurosporine alone had no significant effect (Table 4.2). The effect of 100 nM staurosporine on 5-HT_{2A} receptor-dependent [^{3}H]-AA release was, therefore, determined. As with the inhibitors of the PLD-PAH pathway, staurosporine inhibited 5-HT_{2A} receptor-dependent [^{3}H]-AA release by 50 % (Fig. 4.17). Similar to 5-HT_{2A} receptor-dependent [^{3}H]-2-AG release, the PKC activator PMA failed to have a significant effect on 5-HT_{2A} receptor-dependent [^{3}H]-AA release.

4.3.2. Discussion

Based on the combined results under specific aims 2 and 3, 5-HT_{2A} receptor-dependent AA release requires both the stimulation of the PLD pathway and 2-AG hydrolysis catalyzed by FAAH. The 5-HT_{2A} receptor-dependent AA release pathway appears more complicated than either the 5-HT_{2A} receptor-dependent

PLC or 2-AG pathways. For example, THL failed to inhibit 5-HT_{2A} receptor-dependent AA release at a concentration that was sufficient to inhibit 5-HT_{2A} receptor-dependent 2-AG release, whereas RHC 80267 and MAFP were able to inhibit the release of both lipids. Perhaps THL, through nonspecific effects, compensates for the decreased AA that results from inhibiting 2-AG production and hydrolysis, by stimulating an enzyme that positively regulates AA release via another pathway, e.g. sn2 DGL.

The complete inhibition of 5-HT_{2A} receptor-dependent AA release by MAFP is most likely the combined result of its inhibitory properties at DGL, MGL, FAAH, and PLA_2, all of which can produce free AA directly. This result leads us to a consideration of the complete inhibition of 5-HT_{2A} receptor-dependent AA release by RHC 80267. RHC 80267 selectively inhibits both sn1 and sn2 DGLs. The DGL responsible for 2-AG biosynthesis is sn1-DGL, whereas the DGL responsible for release of free AA is sn2-DGL. Because RHC 80267 inhibits both of these DGLs, a complete inhibition of 5-HT_{2A} receptor-dependent AA release can be explained using the model presented below (Fig. 4.18). The key is that DAG produced following either PLD-PAH or PC-PLC activation, but not PI-PLC activation, is a substrate for sn2-DGL. Based on given data under specific aim 2, DAG produced from either the PC-PLC pathway or the PLD-PAH pathway does not contribute to 2-AG production in the NIH3T3-5HT_{2A} cell line, whereas

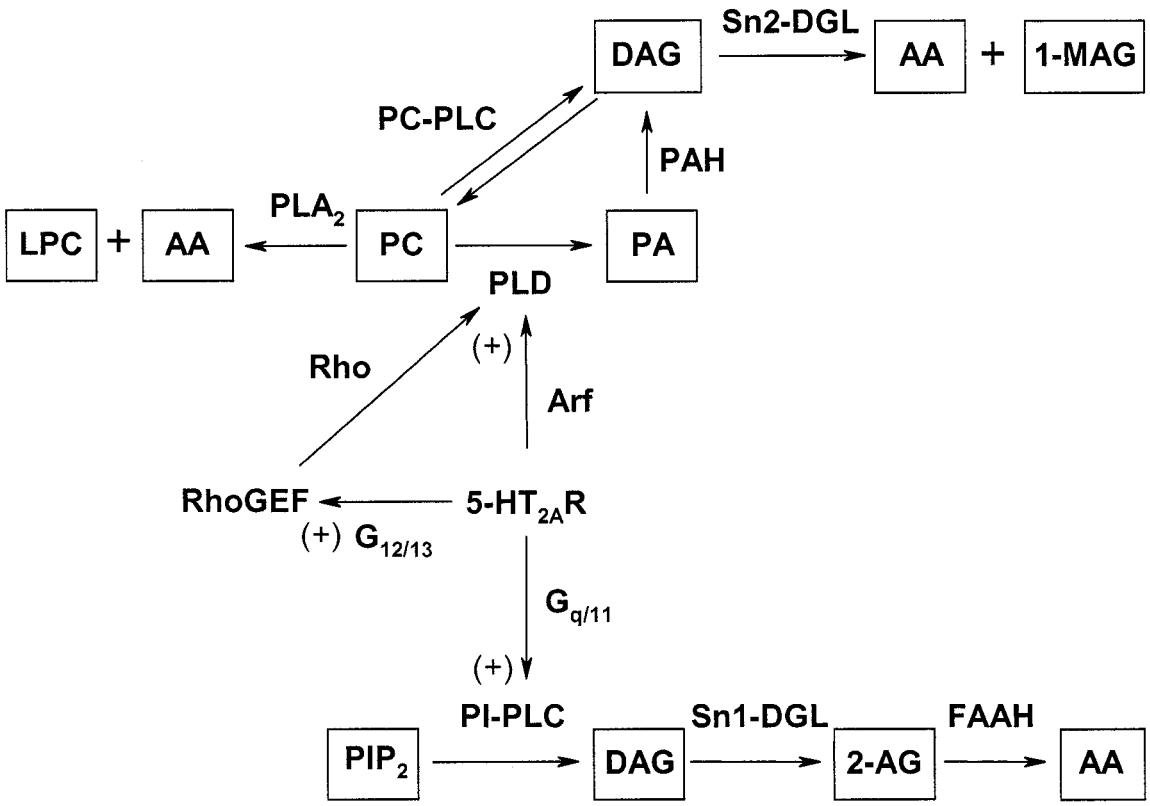


Figure 4.18 Model of 5-HT_{2A} receptor-dependent lipid metabolism. Activation of the 5-HT_{2A} receptor stimulates PI-PLC, PLD, and PLA_2. PI-PLC stimulation leads to 2-AG production from DAG and eventually AA catalyzed by Sn1-DGL and FAAH (or MGL) respectively. PLD stimulation leads to DAG production following PAH-mediated hydrolysis of PA. DAG produced by this pathway may activate PKC or be utilized as a substrate by Sn2-DGL producing AA directly. PKC may play a role in 2-AG hydrolysis and/or uptake. PLA_2 produces AA directly by hydrolyzing PC. PLA_2 activity may be stimulated by a complex signaling network (Kurrasch-Orbaugh *et al.* 2003a) not illustrated here. Lipids are shown boxed whereas enzymes that modify them are illustrated with arrows that indicate the direction of the reaction under stimulated conditions. A (+) sign indicates a stimulation event.

inhibitors of the PLD-PAH pathway generate a small but significant stimulation of 2-AG release over basal, and have zero, or in the case of n-butanol a potentiating, effect on 5-HT-stimulated 2-AG release.

It was speculated above that the PC-PLC pathway and the PLD-PAH pathway stimulate an enzyme that regulates 2-AG production or hydrolysis. It is possible that DAG produced following the activation of these enzymes leads to PKC stimulation that may be a required element in 2-AG hydrolysis. This model explains how both the PLD pathway and FAAH contribute to 5-HT_{2A} receptor-dependent AA release. Although there was no observed inhibition of 5-HT_{2A} receptor-dependent AA release when the PC-PLC inhibitor D609 was used, the PLD-PAH pathway may compensate for the reduced DAG produced from PC-PLC because higher membrane concentrations of PC would be available as a substrate for PLD. According to this model DAG from PI-PLC and DAG from PLD-PAH would be produced in different membrane microdomains. Although none of the experiments outlined in this thesis specifically addressed this hypothesis, there is evidence in the scientific literature to support the idea. For example, PLD (Czarny *et al.* 1999;Yoon *et al.* 2003;Cho *et al.* 2004) and PAH (Nanjundan and Possmayer 2001) have been found localized to Caveolin-1 rich microdomains. Alternatively, PIP_2, the substrate of PLC, has been found to be exclusively localized within the plasma membrane, with little found in Caveolin-1 rich microdomains (Watt *et al.* 2002). Assuming PLC to be localized, along with its substrate PIP_2, outside of caveolae, a case can be made for distinct and independent DAG pools being accessible following 5-HT_{2A} receptor stimulation.

The data presented under specific aims 2 and 3 suggest that PLD activation is a required element in 2-AG metabolism. McFarland et al., (2004) found evidence that endocannabinoid transport and subsequent metabolism are mediated through caveolae-related (clathrin-independent) endocytosis. Interestingly, the 5-HT_{2A} receptor has been found to associate with Caveolin-1 (Bhatnagar *et al.* 2001). Furthermore, both PLD and ARF6 have been shown to be involved in GPCR internalization (Du *et al.* 2004;Houndolo *et al.* 2005). Taken together, these studies support the hypothesis that signaling pathways that trigger internalization of 5-HT_{2A} receptors (ARF and PLD) may be involved in the hydrolysis of 2-AG to AA. The results under specific aim 3 support a role for PLD, PAH, and PKC in this process. Additionally, sn2-DGL may be the enzyme responsible for a part of the direct AA release, considering that the non-specific DGL inhibitor RHC80267 fully inhibited AA release.

Although not an endocannabinoid, AA is likely a retrograde signaling molecule of relevance to 5-HT_{2A} receptor action. At the apical dendrites of pyramidal cells in layer V of the prefrontal cortex, 5-HT, as well as hallucinogenic 5-HT_{2A} receptor agonists, cause an increase in the frequency of EPSCs (Aghajanian and Marek 1997;Aghajanian and Marek 1998;Aghajanian and Marek 1999). An increase in EPSC frequency is characteristic of an increase in presynaptic glutamate release. With the localization of 5-HT_{2A} receptors to postsynaptic dendritic spines and shafts (Miner *et al.* 2003), 5-HT_{2A} receptor-mediated effects on presynaptic terminals are likely the result of a retrograde messenger. Lambe and Aghajanian, (2001) reported that blocking presynaptic

Kv1.2 channels with α-dendrotoxin occluded 5-HT_{2A} receptor-dependent presynaptic glutamate release at thalamocortical terminals in the prefrontal cortex. It is likely that AA or one of its eicosanoid derivatives is the retrograde messenger that is responsible for this process. Future studies should focus on testing this hypothesis.

CHAPTER 5. CONCLUSIONS

From the results of the experiments outlined under specific aim 1, we may be led to conclude that relative 5-HT_{2A} receptor-dependent IP accumulation is a good predictor of the hallucinogenic potency of 5-HT_{2A} receptor agonists. If one looks beyond the phenethylamines, however, to the tryptamines and ergolines, the present data refute this conjecture. For example, upon analyzing a series of ergoline compounds (Appendix) we find that LSD, one of the most potent hallucinogens, is a poor stimulator of IP accumulation. In fact, the piperidide derivative of lysergic acid (LA-Pip), which lacks significant hallucinogenic potency (Cerletti and Doepfner 1958), was found to be a better agonist for IP accumulation relative to LSD (Appendix). If IP accumulation was correlated to hallucinogenesis, one would predict that LA-Pip would be an even more potent hallucinogen than LSD. The tryptamine hallucinogen psilocin is another example that confounds the IP accumulation hypothesis of hallucinogenesis. Psilocin produces hallucinogenic effects in humans at a dosage comparable to the phenethylamine analogues 2C-B, 2C-I, and 2C-T2 (~10-20 mg) (Shulgin and Shulgin 1991;Shulgin and Shulgin 1997), although its EC50 and intrinsic activity for IP accumulation are 2,300 nM and 46%, respectively, versus approximately 20 nM and 50% for the phenethylamines. Considering that on an EC50 basis the

phenethylamine analogues are approximately two orders of magnitude more potent at stimulating IP accumulation than psilocin, one might conclude that their human dose would be much lower relative to psilocin. The inconsistencies observed with regard to hallucinogenic potency and yet weak stimulation of IP accumulation by ergoline and tryptamine hallucinogens essentially refutes the IP accumulation hypothesis of hallucinogenesis.

As suggested in the background section of this thesis, electrophysiological studies support the hypothesis that the effects of hallucinogens are mediated, at least in part, by a retrograde messenger that blocks Kv1.2 channels on presynaptic glutamatergic axon terminals (Lambe and Aghajanian 2001). The results under specific aim 2 conclusively demonstrate that the 5-HT_{2A} receptor can stimulate the production of the retrograde messenger 2-AG. Animal studies provide evidence for 5-HT_{2A} receptor-dependent endocannabinoid release, and indicate that 5-HT_{2A} receptor-dependent endocannabinoid release inhibits behaviors that are associated with 5-HT_{2A} receptor activation (Darmani and Pandya 2000;Darmani 2001;Gorzalka *et al.* 2005), an observation that is not consistent with Kv1.2 channel blockade. Perhaps 5-HT_{2A} receptor-dependent endocannabinoid release simply provides a mechanism for pyramidal cells to regulate negatively the stimulatory activity brought about by postsynaptic 5-HT_{2A} receptor activation, whereas another retrograde messenger facilitates the effects observed in the electrophysiological studies.

Results from animal studies and from experiments conducted for this thesis suggest that stimulation of 5-HT_{2A} receptor-dependent endocannabinoid

release would lead to decreased pyramidal cell activity via endocannabinoids, in direct contradiction of electrophysiological studies showing that 5-HT_{2A} receptor activation *increases* pyramidal cell activity (Marek and Aghajanian 1996;Aghajanian and Marek 1998;Aghajanian and Marek 1999;Marek and Gewirtz 1999;Lambe *et al.* 2000).

Fortunately, there is more to this story than PLC stimulation and 2-AG release. In fact, because 2-AG production is dependent on PLC activity, we know that 2-AG release is probably not correlated with hallucinogenesis. This conclusion leads us to consider 5-HT_{2A} receptor-dependent eicosanoid release. Kurrash-Orbaugh *et al.*, (2003b) demonstrated that hallucinogens tended to favor 5-HT_{2A} receptor-dependent eicosanoid release over 5-HT_{2A} receptor-dependent IP accumulation. For example, LSD, although not a particularly efficient agonist for IP accumulation (9.8 nM, 22% 5-HT) is a better agonist for eicosanoid release (20 nM, 56% 5-HT). Perhaps even more striking are the differences seen with the tryptamine hallucinogens. Both 5-MeODMT and psilocin have EC50s for 5-HT_{2A} receptor-dependent IP accumulation in the 2300-2400 nM range, whereas their EC50s for 5-HT_{2A} receptor-dependent eicosanoid release are in the 100-200 nM range. Relatively non-hallucinogenic unsubstituted tryptamine has comparable EC50s for 5-HT_{2A} receptor-dependent IP accumulation and eicosanoid release in the 1100-1200 nM range, and has an even lower intrinsic activity (41%) for 5-HT_{2A} receptor-dependent eicosanoid release compared to IP accumulation (91%). It should be mentioned that intravenous infusion of 250 mg of tryptamine induced symptoms in man that were "not unlike those seen with

small doses of LSD" (Martin and Sloan 1970), whereas only 2.3 mg of intravenous 5-MeODMT was required to produce a strong hallucinogenic effect (Shulgin and Shulgin 1997). These results are not consistent with the PI hydrolysis data for these compounds that if considered alone would suggest that tryptamine was the more potent hallucinogen.

Considering that: (1) presynaptic Kv1.2 channels are blocked by eicosanoids (Poling *et al.* 1995;Poling *et al.* 1996a;Poling *et al.* 1996b), (2) blockade of Kv1.2 channels occludes the action of hallucinogens on presynaptic glutamate release (Lambe and Aghajanian 2001), and (3) hallucinogens tend to favor eicosanoid release over IP accumulation (Kurrasch-Orbaugh 2002;Kurrasch-Orbaugh *et al.* 2003b), we can conclude that the 5-HT_{2A} receptor-dependent eicosanoid release is likely to be the more relevant signaling pathway for the psychopharmacology of hallucinogens.

Based on the electrophysiological studies of Aghajanian (Marek and Aghajanian 1996;Aghajanian and Marek 1998;Aghajanian and Marek 1999;Marek and Gewirtz 1999;Lambe *et al.* 2000;Lambe and Aghajanian 2001), a model can be constructed to explain what is happening at glutamatergic synapses in the prefrontal cortex when a hallucinogen is present. These models are best understood with the help of Figure 5.1. During normal waking consciousness 5-HT is tonically released into the cortical compartment in pulses corresponding to the firing rhythm of raphe cells, which send ascending projections into the cortex. Concentration waves of 5-HT migrate toward postsynaptic pyramidal cell apical dendrites that are receiving oscillatory

glutamatergic input from the thalamus and adjacent pyramidal cells. Upon binding to 5-HT_{2A} receptors, both eicosanoids and 2-AG are produced in concentrations that depend on the timing of the cycle of postsynaptic glutamate-dependent depolarization. For example, in the case of a simultaneous postsynaptic NMDA receptor-dependent depolarization and 5-HT_{2A} receptor activation, more 2-AG would be produced as a result of the coincidence detecting ability of PLCβ (Hashimotodani *et al.* 2005) than if the stimuli occurred out of phase. It is possible that a similar coincidence detector exists for eicosanoid release, as some isoforms of PLA_2 are known to be calcium dependent (Fawzy *et al.* 1987;Seilhamer *et al.* 1989;Marshall and McCarte-Roshak 1992;Peters-Golden and McNish 1993), and as we saw under specific aim 3, AA release was stimulated by a calcium ionophore.

Following release, the 2-AG migrates to the presynaptic glutamatergic axon terminal where it binds to CB1 receptors, inhibits glutamate release, and is rapidly hydrolyzed by MGL (Dinh *et al.* 2002a;Dinh *et al.* 2002b). Other eicosanoids (e.g. AA) also migrate to the presynaptic terminal and block Kv1.2 channels, resulting in the increase in asynchronous glutamate release observed by Lambe and Aghajanian (2001;2006). Because 2-AG and AA arise from different signaling pathways, it is likely they are released on different time scales and would therefore not elicit their effects simultaneously on the presynaptic neuron. It is assumed that under the conditions of normal waking consciousness

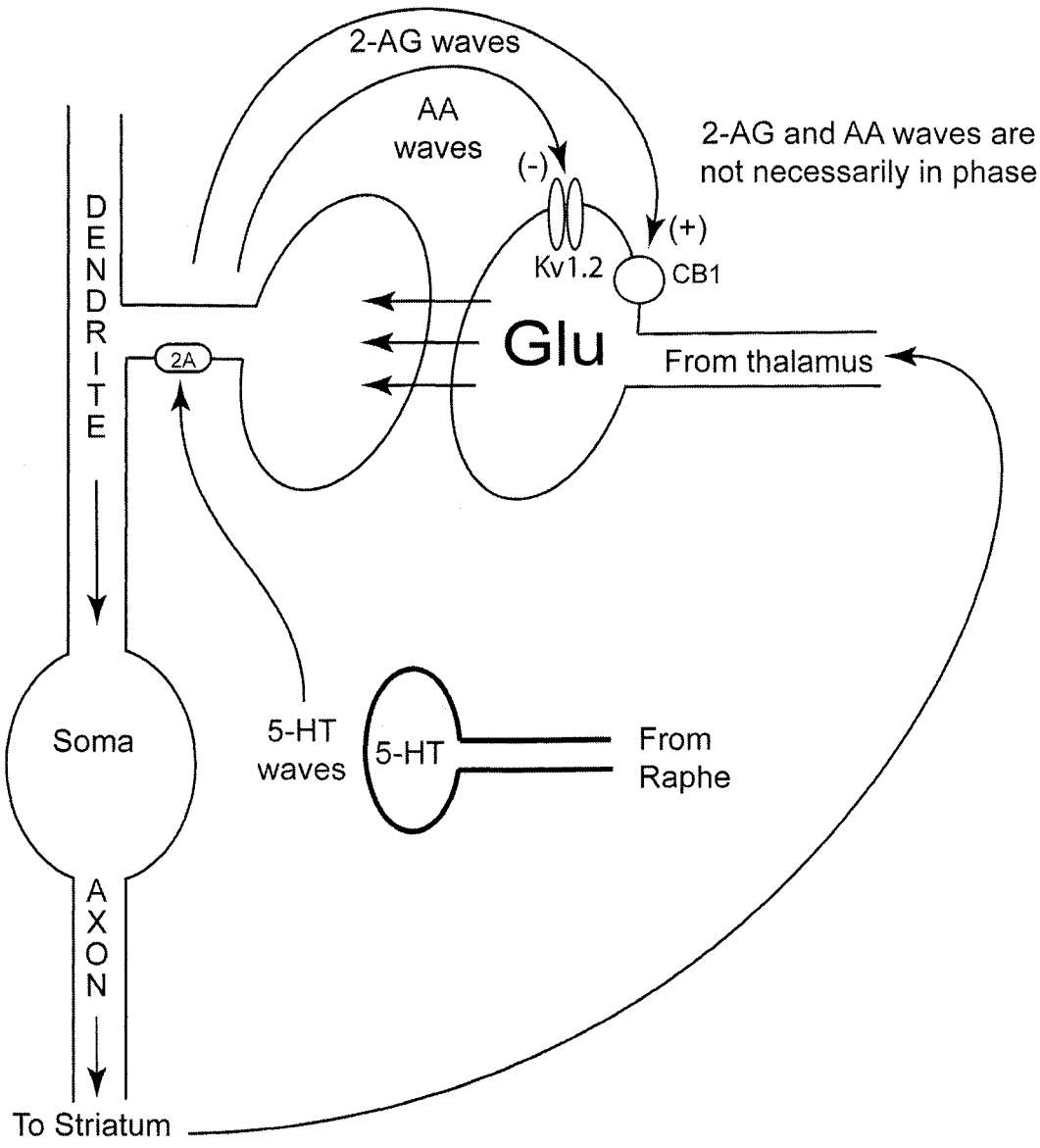


Figure 5.1 How 5-HT influences the cortical component of a CSTC oscillation. Activation of the 5-HT_{2A} receptor stimulates the release and retrograde transport of 2-AG and AA that influence the presynaptic neuron of a thalmo-cortical glutamatergic synapse. As hallucinogens favor AA release over PLC activation and 2-AG release, the diffusion waves of the two lipids would differ relative to those produced under normal 5-HT stimulation.

some rhythm or equilibrium frequency of concentration oscillations for 5-HT and all of the lipid messengers is maintained in the synapse, with allowance for changes in attention of the conscious individual. This model only considers the cortical component of a cortico-striato-thalamo-cortical (CSTC) oscillation (Vollenweider and Geyer 2001).

When a hallucinogen is introduced into the system, the CSTC oscillations may be disrupted in the following ways. First, we know that all three of the hallucinogen classes lead to inhibition of raphe cell firing (Aghajanian *et al.* 1968;Aghajanian *et al.* 1970;Aghajanian 1972;Aghajanian and Haigler 1975;deMontigny and Aghajanian 1977), effectively disrupting 5-HT release within the intracortical space. Second, hallucinogens disrupt the balance of retrograde signaling molecules in favor of eicosanoids over 2-AG, effectively decreasing negative feedback inhibition by the endocannabinoid, while at the same time increasing the blockade of Kv1.2 channels, leading to asynchronous glutamate release. The disruption of the CSTC oscillations that maintain normal waking consciousness temporarily breaks down the integrity of cortical processing and leads to the frontal cortical hyperactivity observed in PET studies of human subjects under the influence of psilocybin (Vollenweider *et al.* 1997).

The biggest limitation of this model is that it does not take into account the entire brain or any of the other brain regions known to be critical for normal cognition, such as the thalamus, where 5-HT_{2A} receptors are densely expressed. The model also does not address the role of 5-HT_{2A} receptors on GABAergic interneurons within the cortex, although the indiscriminate stimulation of

GABAergic interneurons by hallucinogens would also contribute to cortical disequilibrium. What the model does attempt to demonstrate, however, is a way that hallucinogens can mediate the physiological effects so far observed in the cortex. Because there are only limited data concerning 5-HT_{2A} receptors in other areas of the brain it would not be prudent to speculate on their possible function(s).

In conclusion, 5-HT_{2A} receptor-dependent IP accumulation and subsequent 2-AG release, as well as 5-HT_{2A} receptor-dependent eicosanoid release, are probably involved in the timing maintenance of the oscillatory behavior of glutamatergic synapses in the cortex. Hallucinogens, via disruption of raphe cell firing and agonist-directed trafficking at the 5-HT_{2A} receptor, may disrupt the normal CSTC oscillatory cycles, thus reducing the stability and integrity of the cortex, leading to the observed hyperactivity and hallucinogenic effects (Vollenweider *et al.* 1997). Future electrophysiological studies should either confirm the results and speculations provided in this thesis or allow them to be refined as further information accumulates.

CHAPTER 6. FUTURE DIRECTIONS

The main assets of this thesis are the testable hypotheses that were revealed upon completion of the experiments in heterologous expression systems. We now know that hallucinogens, through stimulation of the 5-HT_{2A} receptor, cause the release of the endocannabinoid 2-AG, at least in heterologous expression systems. In order to confirm the significance of this finding it will be necessary for electrophysiological experiments to be preformed in prefrontal cortical slices. For example, it would be most enlightening to examine the effect on EPSCs in pyramidal neurons pretreated with a CB1 antagonist and subsequently stimulated with a 5-HT_{2A} agonist. We would expect to observe a potentiation of 5-HT_{2A} receptor-dependent EPSCs. Another experiment could use a MGL inhibitor in order to prevent the metabolism of endogenously produced 2-AG following subsequent 5-HT_{2A} receptor stimulation. In that case, we would expect to observe a reduction or abolition of 5-HT_{2A} receptor-dependent EPSCs. The same result would be predicted if pyramidal neurons were treated with a CB1 agonist. The introduction of either a DGL inhibitor or a PI-PLC inhibitor into this experimental system would be expected to potentiate 5-HT_{2A} receptor-dependent EPSCs, a result that would not be intuitive

because PI-PLC activation is presently considered to be the main 5-HT_{2A} receptor-dependent response.

Electrophysiological experiments addressing 5-HT_{2A} receptor-dependent eicosanoid release also are warranted. For example, it would be interesting to determine whether AA or any of its metabolites occlude the asynchronous release of glutamate from presynaptic terminals as was demonstrated for α-dendrotoxin (Lambe and Aghajanian 2001;Lambe and Aghajanian 2006).

Future animal studies could be done to confirm the results of Gorzaka *et al.* (2005). For example, would the preadministration of the MGL inhibitor URB602 lead to an inhibition of 5-HT_{2A} receptor-mediated WDS in rats?

Furthermore, it would be useful to identify the other metabolites produced during 5-HT_{2A} receptor-dependent eicosanoid release and determine whether or not any of them have unique effects on presynaptic glutamate release. In the rat brain, eicosanoid release is robustly stimulated in many brain regions following treatment with the 5-HT_{2A} receptor agonist DOI (Qu *et al.* 2003).

Also, it would be interesting to determine whether a selective CB1 agonist could block the subjective hallucinogenic effects of psilocybin in humans or the cortical hyperactivity seen in PET scans of humans under the influence of psilocybin (Vollenweider *et al.* 1997). It should be noted that tetrahydrocannabinol would not be a suitable drug for this study as it blocks Kv1.2 channels similar to anandamide (Poling *et al.* 1996a), which may explain why antidotal reports suggest that marijuana does not significantly inhibit the subjective effects of hallucinogens in man (www.erowid.org 2006).

With regard to 5-HT_{2A} receptor signal transduction, it would be useful to characterize further the role of calcium in the release of 2-AG and eicosanoids. It would be necessary to change the composition of the assay medium in order to undertake experiments studying the role of calcium, because the assay buffer used in all of the 2-AG and eicosanoid release experiments in this thesis utilized a concentration of extracellular calcium that was too high to chelate it effectively with reasonable concentrations of BAPTA. Perhaps KRH buffer with 2.2 mM calcium ion, used by researchers studying endocannabinoid uptake (McFarland *et al.* 2004), would be more appropriate.

Some targets that remain unchallenged in this thesis are the DAG-activated TRPC channels as well as the calcium channels located on the ER membrane. It is likely that activation of these channels plays a role in calcium signaling and the stimulation of eicosanoid and/or 2-AG release. Determining the effects of calmodulin antagonists on 5-HT_{2A} receptor-dependent eicosanoid/2-AG release would also be interesting, as at least one study (Arvanov *et al.* 1999) has suggested a role for calmodulin and downstream effectors in cortical 5-HT_{2A} receptor signal transduction.

Finally, it may be useful to assess the relative stimulation by 5-HT_{2A} receptor agonists of the eicosanoid and PLC effector pathways in order to determine whether or not the eicosanoid release pathway is correlated with the subjective hallucinogenic effects in humans. Although not mentioned specifically in the results section of this thesis, a few potent 5-HT_{2A} receptor agonists have been discovered to favor IP accumulation preferentially over eicosanoid release

(See (McLean *et al.* 2006) and Appendix). Based on the model presented in this thesis, we would predict that these compounds are not hallucinogenic, although it is unlikely that this hypothesis will ever be tested in humans. It may be useful, however, to examine the ability of other known non-hallucinogenic 5-HT_{2A} receptor agonists to stimulate eicosanoid release. An example of such a compound would be the piperidine amide of lysergic acid (Cerletti and Doepfner 1958).

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APPENDIX

APPENDIX

LSD derivatives

A series of LSD derivatives was characterized for competition binding and IP accumulation relative to LSD (Fig. A.1). The LA-Pip derivative was approximately a third as potent as LSD but had roughly twice the intrinsic activity of LSD for IP accumulation. Although the LA-Pip derivative has roughly twice the affinity relative to LSD for the human 5-HT_{1A} receptor, its 5-HT_{2A} and 5-HT_{2C} receptor affinities are comparable Curiously this compound is inactive in man (Cerletti and Doepfner 1958).

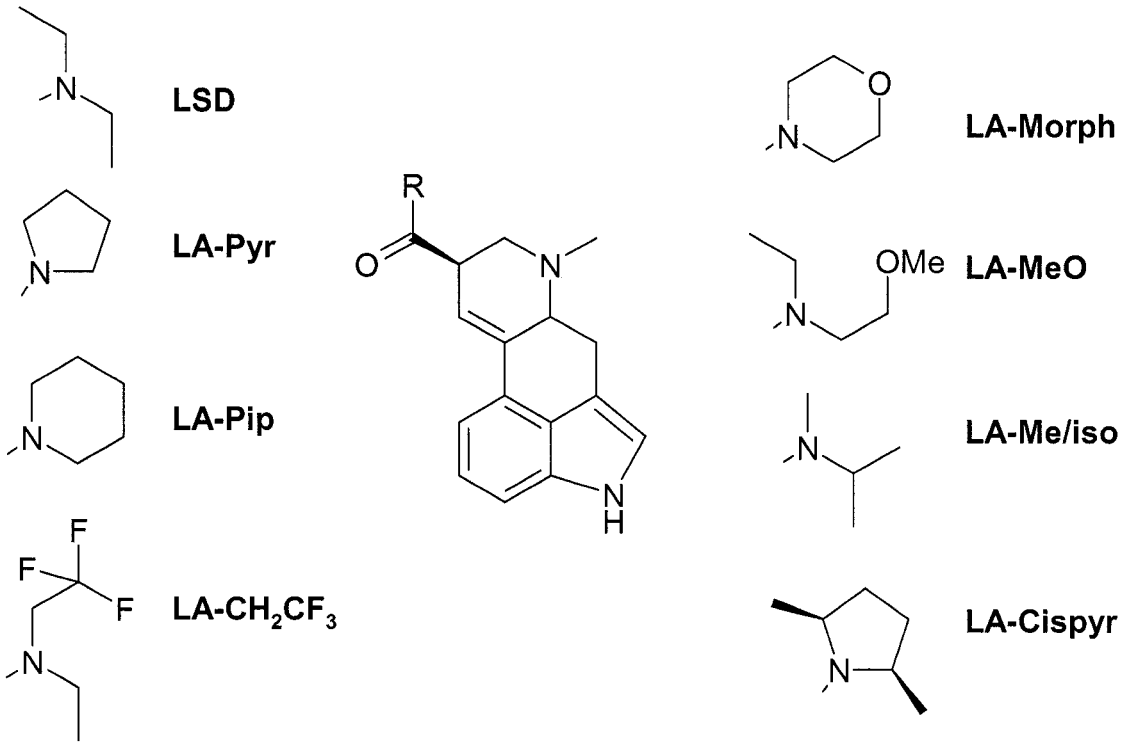

Figure A.1 The series of amide substituted LSD derivatives used in this study.

Table A.1 Results of competition binding experiments and IP accumulation assays for the series of ergolines illustrated in Figure A.1. K_is and EC50s are expressed in nM with SEM in parentheses. Intrinsic activity (Int. Act.) is expressed as the percent stimulation relative to a basal and 5-HT control with SEM in parentheses.

	Binding Affinity (K_i)			PI hydrolysis (r5-HT_{2A})	
DRUGS	**r5-HT_{2A} [^{125}I]-DOI**	**r5-HT_{2C} [^{125}I]-DOI**	**h5-HT_{1A} [^{3}H] 8-OH-DPAT**	**EC50**	**Int. Act**
LSD	2.10 (0.03)	2.3 (0.2)	1.1 (0.3)	8.4 (1.6)	22 (3)
LA-Pyr	12.2 (0.2)	6.1 (0.5)	0.66 (0.08)	140 (26)	34 (5)
LA-Pip	2.6 (0.1)	2.3 (0.1)	0.45 (0.09)	26 (2)	46 (6)
LA-CH_2CF_3	1.6 (0.03)	1.8 (0.2)	1.1 (0.2)	19 (3)	33 (4)
LA-MorphI	16 (2)	51 (2)	2.6 (0.3)	173 (35)	32 (3)
LA-MeO	7.1 (0.4)	7.8 (0.5)	4.0 (0.9)	30 (7)	30 (5)
LA-Me/iso	3.2 (0.1)	7.4 (0.4)	2.1 (0.4)	18 (4)	36 (2)
LA-Cispyr	27 (1)	11.2 (0.5)	24 (5)	18 (4)	37 (4)

N-benzylphenethylamines

A new series of extremely potent phenethylamine derived 5-HT_{2A} receptor agonists was characterized for competition binding affinity and functional assays. The *N*-benzylphenethylamines represent some of the most potent 5-HT_{2A} receptor agonists (Fig. A.2). Although these compounds are potent stimulators of 5-HT_{2A} receptor-dependent IP accumulation, they are less effective in stimulating 5-HT_{2A} receptor-dependent eicosanoid release (Table A.2). On an EC50 basis, the o-fluorobenzyl (Q) derivative was 23 fold selective for the PLC pathway over eicosanoid release. Because hallucinogens tend to be selective for eicosanoid release (Kurrasch-Orbaugh 2002;Kurrasch-Orbaugh *et al.* 2003b), it is possible that these compounds are not hallucinogenic.

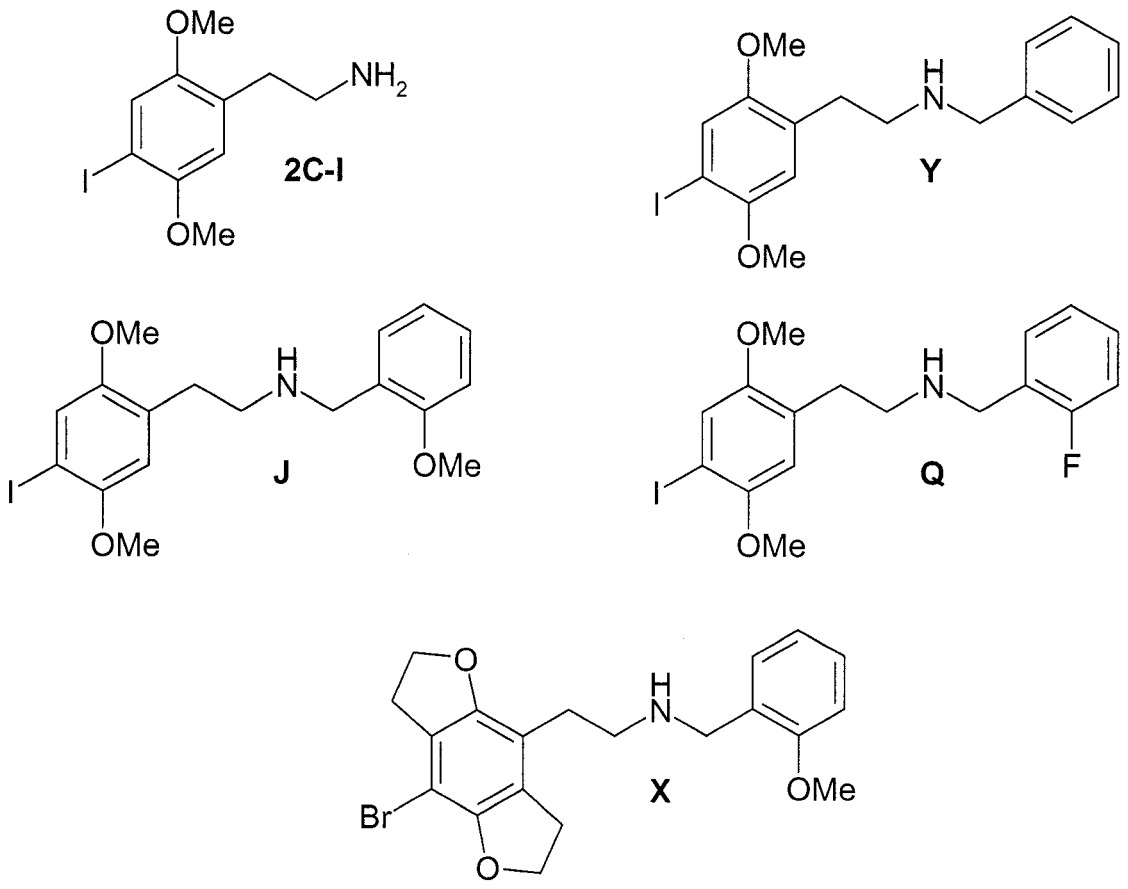

Figure A.2 The IP accumulation-selective *N*-benzylphenethylamine class of 5-HT_{2A} receptor agonists

Table A.2 Results of competition binding experiments and functional assays for the series of *N*-benzylphenethylamines illustrated in Figure A.2. K_is and EC50s are expressed in nM with SEM in parentheses. Intrinsic activity (Int. Act.) is expressed as the percent stimulation relative to a basal and 5-HT control with SEM in parentheses.

	K_i [^{125}I]-DOI		PI hydrolysis		Eicosanoid Release	
DRUGS	**r5-HT$_{2A}$**	**r5-HT$_{2C}$**	**EC50**	**Int. Act**	**EC50**	**Int. Act**
2C-I	0.65 (0.12)	nd	19 (3)	59 (4)	23 (3)	25 (4)
J	0.09 (0.01)	0.13 (0.02)	2.5 (0.5)	78 (6)	8.0 (0.4)	49 (3)
Y	0.31 (0.03)	0.90 (0.15)	12.0 (0.7)	37 (2)	198 (25)	38 (7)*
Q	0.28 (0.04)	0.85 (0.11)	23 (1)	32 (3)	530 (51)	27 (2)
X	0.14 (0.03)	0.26 (0.05)	1.29 (0.09)	42 (1)	30 (3)	54 (8)

VITA

VITA

Jason Charles Parrish was born on February 8, 1977 to Leslie and Pamela Parrish in Weymouth, Massachusetts. After barely graduating from Hanover High School (Hanover, MA) in 1995, he headed south of the border to attend the University of South Alabama in Mobile, AL as a biology major. It was here that Jason fell in love with his resident advisor Adriane C. Cej and, following her graduation in 1998, the couple decided to move to the Pacific Northwest. Jason began enrollment at the University of Idaho as a molecular biology and biochemistry major. It was there that Jason discovered that organic chemistry was not as it had been described to him, but was in fact most useful in the understanding of biology. Upon graduating *summa cum laude* in December 2000, Jason was accepted into the Medicinal Chemistry and Molecular Pharmacology graduate program at Purdue University where he attempted to gain a scientific appreciation for some of the pharmacological curiosities that he had encountered over the years. In August of 2006 with his love of Belgian beer in mind, Jason accepted an employment offer as the quality control manager for Brewery Ommegang in Cooperstown, NY.